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CYTOLOGICAL ABNORMALITIES PRODUCED BY EXPERIMENTAL TEMPERATURE SHOCK ON TRITURUS TOROSUS EMBRYOS[1]

CATHERINE HENLEY

University of North Carolina

INTRODUCTION

The importance of a complete chromosome complement in normal development is obvious when one considers the evidence that the chromosomes are the carriers of genes governing the characters of the organism. It is of considerable theoretical and practical interest to ascertain the effects of addition or loss of single chromosomes, or of chromosome sets.

Most of the early work in the field of developmental genetics was done with plants, until publication of the reports of Fankhauser (1938 and subsequent papers). These reports indicated the value of a tail-tip method for the cytological study of amphibian larvae. The technique utilized is sufficiently simple that systematic studies can be carried out on a large scale, to determine the incidence of heteroploidy in a natural population of salamander larvae. It was with the view of making such a study on *Triturus torosus* that investigations were begun in 1947 on larvae developing from eggs which had been shipped to Chapel Hill from California, in a mixture of ice and water. These larvae showed a very high incidence of mosaic heteroploidy (43 per cent in a sample of 126 larvae), and of severe mitotic abnormalities of various types. The results of this study will be presented in a separate paper.

The possibility existed that such a frequent occurrence of mosaicism was present in a natural population, as well as in the shipped material. Cytological study of a large group of *T. torosus* larvae collected in nature as young embryos and raised in the laboratory indicated that this was not so (Costello and Henley, 1948, 1949). It was further demonstrated that larvae raised from eggs collected in very cold water had a higher incidence of mitotic abnormality than those collected from warmer water. This finding, coupled with similar work reported by Böök (1943, 1945) and Barber and Callan (1943) implied that low temperature might be at least one of the factors responsible for the effects observed in the shipped embryos. Experiments were therefore planned to test the effects of cold treatment applied at various stages of development, from a period shortly after insemination through the tail-bud stage, in *T. torosus.*

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[1] A dissertation submitted to the Faculty of the University of North Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology.

Some of the data included here were communicated earlier in a preliminary note (Henley, 1948).

The study was carried on under the direction of Dr. D. P. Costello, to whom the author is very grateful for his stimulating interest and his helpful criticisms. The experiments were performed at the School of Biological Sciences of Stanford University during the winter and spring of 1948; it is a pleasure to acknowledge the many courtesies extended during that time by Dr. Douglas M. Whitaker, and Dr. Victor C. Twitty and his co-workers. The accompanying photomicrographs were made by Dr. Costello.

METHODS

The embryos used in these experiments were raised in the laboratory at Stanford University from *Triturus torosus* eggs collected at Los Trancos and Ukiah, California, and from eggs laid in the laboratory by female salamanders collected at Ukiah, Portola and Mt. Hamilton. In all cases, the embryos treated in 1948 were raised within a constant temperature range (13 – 15° C.) except during the experimental treatment. Usually, several egg clutches were treated simultaneously, in a large fingerbowl. Shortly before the time of hatching, the egg masses were segregated, so that embryos from a given clutch could be identified. In the 1948 experiments, non-chlorinated Searsville Lake water was used and the larvae were isolated in separate paraffined paper ice-cream cups according to the method described by Costello and Henley (1949). All animals were photographed at the time of the first clipping (Fig. 1). MS-222 (1:2000 in distilled water) was used for anesthetizing the larvae for photography and tail-clipping.

All the 1948 cold treatments reported here involved a temperature shock. Embryos at various stages of development from before cleavage through tail-bud stages were placed in fingerbowls of pre-chilled water maintained at the desired temperature. At the end of the treatment, they were transferred back to the original fingerbowl of water at room temperature and allowed to continue development. The effects of temperatures from 0° (mixture of ice and water) to 8° C. were tested for varying periods of time, as indicated in Table 1. In all cases, the embryos were left within the jelly mass until they hatched normally.

Appropriate controls were kept for the various series; details from the results of a study of these untreated larvae are discussed by Costello and Henley (1949).

When the larvae had hatched and feeding was about to begin, they were photographed and assigned serial numbers. The distal one-third of the tail-tip was clipped, fixed and stained as described by Costello and Henley (1949). After an interval of approximately 12 days, the regenerated tip was likewise amputated and prepared for cytological study. These "second" tips are far superior to "first" tips, since yolk resorption is completed and more mitoses are usually present. All tips were stained with Harris's acid haematoxylin.

Proper feeding is essential if good cytological preparations are to be obtained; an adequate supply of Artemia nauplii was kept in the cups with the larvae to serve as food. These nauplii were cultured in a strong brine solution and washed into fresh lake water before being fed to the salamander larvae.

I am indebted to Dr. Costello for the use of 27 tail-tip preparations of *T. torosus* larvae, made by him in 1942. These tips were derived from embryos which had been cold-treated 5 to 80 minutes after insemination, for periods of 18 to 23 hours. No preparations from newly regenerated tail-tips of this group were prepared and only first clippings are included in the data. The larvae were raised at laboratory room temperature. The cold treatments did not involve temperature shock, the eggs having been chilled gradually and allowed to return slowly to room temperature.

The tail-tip epithelium was studied at a magnification of 210 ×, and any figures of special interest noted on a small "map" of the tip. Using an oil immersion objective and 10 × compensating occulars, camera lucida drawings of nuclear size were then made of an area on the dorsal side of each tip, as described by Costello and Henley (1949). Mitotic figures were drawn with the camera lucida at a magnification of 2100 ×, in order to establish chromosome counts.

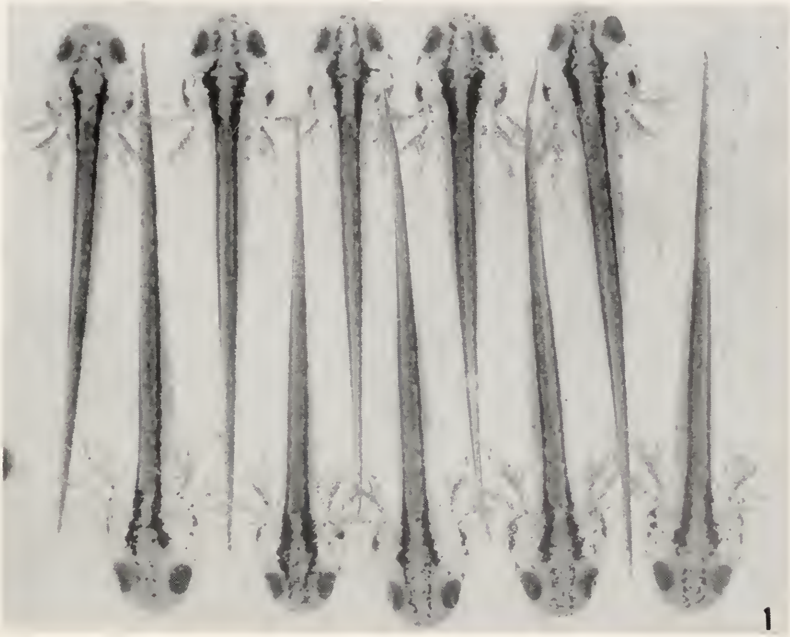


FIGURE 1. A group of *T. torosus* larvae, cold-treated at the gastrula stage for 2 hours at 0° C.; photographed just before their tail-tips were clipped the first time. All ten individuals are normal in general morphology and pigment pattern, but cytologically abnormal to varying degrees. Magnification: 4.5 ×.

Three criteria were utilized in classifying the tail-tips: variations in nuclear size or nucleolar number, the presence of abnormal mitotic configurations, and the presence of heteroploid metaphases. The relative value of these criteria has been discussed in the paper of Costello and Henley (1949); they conclude that only decisive chromosome counts of clear mitotic figures are reliable for a definite diagnosis of chromosomal mosaicism, but point out that nucleolar number and nuclear size

TABLE I

The incidence of cytological abnormalities in tail-tip preparations of T. torosus larvae subjected to cold at various stages of development

Stage treated	Treatment	First tips					Second Tips				
		No. tips	Diploid	Variable nuclei	Abnor. mitoses	Mosaics	No. tips	Diploid	Variable nuclei	Abnor. mitoses	Mosaics
10 min. a.i.*	0°–45 mins.	12	4 33%	5 42%	3 25%	0 0	7	0 0	5 71%	2 29%	0 0
Cleavage, blastula	0°–4 hrs.	115	51 44%	49 43%	14 12%	1 0.9%	82	15 18%	28 34%	34 41%	5 7%
Blastula	0°–1 hr.	28	4 14%	11 39%	12 43%	1 4%	20	3 15%	7 35%	6 30%	4 20%
Blastula	8°–3 days; 5°–12 days	10	3 30%	7 70%	0 0	0 0	4	1 25%	0 0	3 75%	0 0
Gastrula	0°–2 hrs.	8	4 50%	2 25%	2 25%	0 0	1	0 0	1 100%	0 0	0 0
Gastrula	0°–2 hrs.	35	10 29%	11 31%	14 40%	0 0	33	5 15%	2 6%	24 73%	2 6%
Neurula	0°–3 hrs.	30	12 40%	13 43%	4 13%	1 3%	3	2 67%	0 0	1 33%	0 0
Neurula	4°–2 hrs.	4	1 25%	3 75%	0 0	0 0	3	0 0	2 67%	1 33%	0 0
Tail-bud	0°–4 hrs.	150	64 43%	55 37%	30 20%	1 0.6%	141	45 32%	62 44%	31 22%	3 2%
Totals		392	153	156	79	4	294	71	107	102	14
Percentages			39%	40%	20%	1%		24%	36%	35%	5%
Controls		582	423	105	53	1	429	284	88	55	2
Percentages			73%	18%	9%	0.17%		66%	20%	13%	0.5%
1942 Material:											
A. 5 min. a.i.*	4°–23 hrs.	5	0	5	0	0					
B. 20 min. a.i.*	0°–1½ hrs.; 4°–19 hrs.	6	1	1	4	0					
C. 30 min. a.i.*	4°–19 hrs.	3	2	1	0	0					
D. 80 min. a.i.*	4°–18 hrs.	13	2	4	2	5					
Totals		27	5	11	6	5					

* "a.i." After insemination.

are often of considerable value as adjuncts to chromosome counts in classifying a given tail-tip. Unfortunately, reliable chromosome counts are often very difficult to make, especially in the experimentally cold-treated material reported here. Early observations on shipped embryos (Costello and Henley, 1947) have indicated that the occurrence of mitoses having a deficiency or a superfluity of single chromosomes may be quite high. Such figures are often crowded, which makes the detection of aneuploid mitoses difficult.

Sometimes tail-tips had more than one type of abnormality; in these cases, classification was based on the most definitely radical of the atypical features. For example, a tip having both marked variation in nuclear size and nucleolar number, and anaphases with lagging elements was classed with the larvae showing abnormalities of mitosis. Similarly, a tip with variable nuclear size, abnormal mitoses and some metaphases having demonstrably more or less than the diploid number of 22 chromosomes (Henley and Costello, 1947) was classified among the mosaic individuals.

RESULTS

The results of a study of 392 "first" tips and 294 regenerated tips are summarized in Table 1, together with the findings obtained from cytological examination of the control tips. No polyploid individuals were observed in either the control or the experimental groups. Among the "first" tips, 153 (39 per cent) were normal diploids, 156 (40 per cent) had nuclei of variable size or abnormalities of nucleolar number, 79 (20 per cent) had abnormal mitoses, and 4 (1 per cent) were chromosomal mosaics. All four of these mosaics appeared to be diploid triploid individuals, although confirmation by chromosome counts was possible in only one case. In the remaining three, it was obvious that more than 22 chromosomes were present in some of the metaphases. Of the 294 regenerated tips, 71 (24 per cent) were clearly diploid, 107 (36 per cent) had nuclei of variable size or variations in nucleolar number, 102 (35 per cent) had abnormal mitoses, and 14 (5 per cent) were mosaics. Included among the mosaics were four diploid/hyperdiploid individuals, seven diploid/triploids, one diploid/triploid/tetraploid, one possible diploid/tetraploid, and one hypodiploid/diploid hyperdiploid/triploid. Five of these diagnoses could be confirmed by chromosome counts, and photographic records were made of two more.

Among the "first" tips, the incidence of all types of abnormality was generally lower than among the "second" tips; this was also found to be true of the control animals. The incidence of abnormality in the experimental group was, in almost every case, double or more than double that found among the controls.

The effectiveness of the various treatments in producing mosaicism and other abnormalities is shown in Table 2, where the data are grouped according to the stage treated, without respect to the temperature or duration of treatment. It appears that the early stages of development, through gastrulation, are somewhat more sensitive to the effects of temperature shock than neurulae and tail-buds. This is especially evident in the regenerated "second" tips.

"First" tips from 27 larvae experimentally subjected in 1942 to low temperature were also studied. These had all been cold-treated within 80 minutes after insemination for relatively long periods of time (18–23 hours). Five of the larvae (19 per cent) were normal diploids, 11 (41 per cent) had nuclei of variable size or abnormalities in nucleolar number, 6 (22 per cent) had abnormal mitoses, and 5 (19 per cent) were chromosomal mosaics. Included among these mosaics were three diploid/triploids, one diploid/triploid/tetraploid, and one diploid/triploid/pentaploid. All except a single diploid/triploid were confirmed by direct chromosome counts.

The variation in nuclear size and nucleolar number noted in these experimentally cold-treated larvae was considerably more pronounced, both qualitatively and quantitatively, than in the control tips. It is of interest to note that there was no direct correlation between nuclear size and nucleolar number. Many of the nuclei within the normal range of size had three or even more nucleoli. Conversely, very large nuclei often had only two nucleoli.

In many of the heteroploid and a few of the diploid metaphases the chromosomes were apparently split (Fig. 6). When counts were made of such figures, each pair of chromatids was counted as a unit.

TABLE II

The percentage of cytological abnormalities found in tail-tip preparations of T. torosus larvae subjected to cold at various stages of development

Stage treated	First tips				Second tips			
	2 N	Var. nuc.	Abn. mit.	Mos.	2 N	Var. nuc.	Abn. mit.	Mos.
10 mins. a.i.*	33%	42%	25%	0	0	71%	29%	0
Blastula	38%	44%	17%	1%	18%	33%	40%	8%
Gastrula	33%	30%	37%	0	14%	9%	70%	6%
Neurula	38%	47%	12%	3%	33%	33%	33%	0
Tail-bud	43%	37%	20%	0.06%	32%	44%	22%	2%
1942 Material:								
5 to 80 mins. a.i.*	19%	41%	22%	18%	—	—	—	—

* After insemination.

Cell condensations which appear identical with the lateral line organs were in an atypical position in many of the preparations. Usually, no visible deficiency was present in the normal arrangement, so that the displaced condensations may represent supernumerary lateral line organs.

Blister-like protuberances were occasionally observed on interphase nuclei; in some instances, blebs of chromatin material appeared to have been extruded from nearby nuclei. These blebs retain the organized appearance of normal complete nuclei, and are thus distinct from the nuclear débris described by Costello and Henley (1949). Abnormal nuclei, similar to those observed in control larvae collected from very cold water, were present in some of the experimentally cold-treated tail-tips. Such nuclei are characterized by the presence of a highly basophilic periphery, surrounding a relatively homogeneous central region which stains less intensely.

Among the more striking mitotic abnormalities observed were masses of relatively uncondensed chromatin, sometimes occurring as isolated configurations, and occasionaly in conjunction with metaphase mitoses. In a few cases, these large masses of basophilic material appeared to be in prophase. An example of this type is shown in Figure 2; here the chromatin mass is connected by a very thin bridge to an adjoining atypical prophase figure, and has many lobes. Three dark bodies (not visible in the photomicrograph) are present in the main mass of chromatin. It is possible that these represent nucleoli, although they do not have the usual appearance of such structures. Normally, nucleoli are not visible in prophase configurations in these tail-tips. In some instances, a chromatin mass in association with a metaphase figure appeared to represent uncondensed chromosomes of the figure, since only a very few normal chromosomes were present.

Metaphase plates occurred in which one or more chromosomes were abnormally oriented on the spindle. Sometimes this abnormality of orientation was so pronounced as to indicate that the chromosome or chromosomes had completely lost spindle connections and were loose in the cytoplasm. Fusion of metaphase chromosomes was apparent in several tail-tips; various steps in the process of fusion were noted, from a stage at which the identity of most of the components was still established to one where only a mass of chromatin was visible. Such chromatin masses are distinguished from the type shown in Figure 2 by the fact that they retain the characteristic ring-shape of a normal metaphase in polar view. Furthermore, the chromatin is much less diffuse than in the unorganized masses of basophilic material. Similar fused configurations were observed in what appear to be telophase (Fig. 9) and anaphase mitoses.

By far the largest number of mitotic abnormalities was observed in anaphases and telophases, confirming an observation made on untreated control material. One of the common types of aberration is shown in Figure 10, where connections persist between several chromosomes of the separating elements. A less radical "sticky" configuration is shown in Figure 11, where only a single connection is present. In the anaphase shown in Figure 12, one of the chromosomes or chromosome fragments appears to be lagging and atypical in its orientation on the spindle. Other examples of this type of abnormality have also been noted. In one case, an apparently normal chromosome is lying at right angles across the spindle; it appears to have lost its connection entirely. Similar examples of "lost" and atypically oriented chromosomes or chromosome fragments are shown in Figures 13, 14, and 18. The fragments may be incorporated into one of the daughter cells as in Figure 13, or lie on the spindle between the two poles as in Figure 14. In the former case, they might contribute to the formation of an aneuploid cell if complete chromosomes are involved. However, in cases where the chromosome material is between the two poles, it seems likely that eventually it would be expelled into the cytoplasm. An abnormal orientation of anaphase poles is shown in Figure 15; one group of daughter chromosomes is lying with the center of orientation almost at right angles to that of the other group. The spindle area of this anaphase shows clearly the characteristic staining properties noted at this stage. Ordinarily, during all stages of mitosis except anaphase and early telophase, only the chromosomes and chromatin material stain blue, leaving the background completely colorless. However, at anaphase, the region between the groups of daughter chromosomes often appears clearly defined as a light blue area. Recognizable spindle fibres have not been definitely identified in the present material using this staining technique, although they seem to be discernible in some anaphase configurations.

The connections between separating anaphase and telophase groups may persist, so that in some preparations, a high percentage of interphase nuclei with connecting "bridges" is present. Occasionally this connection is thin and attenuated; in other instances it is a rather substantial structure. All gradations between the two extremes were noted, as well as cases where the thin connection appears to have been broken, leaving only a slender projection from each daughter nucleus. Sometimes persistent connections have very sharp bends at the middle, as though some localized pressure were being exerted at that point. In many cases of connected daughter nuclei, the components are asymmetrical, so that one pole is normal in size or larger than normal, while the other is considerably smaller.

In some tail-tips, anaphases or telophases were observed in which nuclear débris, of the type described by Costello and Henley (1949) is present on the spindle or incorporated into one of the daughter nuclei. The characteristic globules of the débris are usually surrounded by a clear halo, which appears well defined against the light blue background of the spindle area.

One remarkably clear tripolar anaphase was found in a tail-tip from a larva cold-treated at the blastula stage (Fig. 16). In this figure, two of the poles appear considerably smaller than the third. The chromosomes in all three groups seem to

---

PLATE I

Figures 2–18 are photomicrographs of chromatin configurations in the tail-tip epithelium of *T. torosus* larvae; from whole-mount preparations stained with Harris's acid haematoxylin. Magnification: 1090 ×.

FIGURE 2. A large mass of chromatin which appears to be in prophase. Two lobes are shown connected to the main mass, at the bottom of the photomicrograph. The connection of the lobe at the left is rather thick, while that of the lobe at the right is quiet slender. From a larva treated at the tail-bud stage for 4 hours at 0° C.

FIGURE 3. Normal diploid metaphase in polar view, with 22 chromosomes readily countable. Compare with Figures 4–9.

FIGURE 4. Triploid (or possibly tetraploid) metaphase. The figure is too crowded for an accurate count, but it is obvious that far more than the normal diploid number of chromosomes is present. From a larva treated at the gastrula stage for 2 hours at 0° C.

FIGURE 5. Triploid metaphase. Twenty-eight chromosomes could be counted accurately and more were present. From a larva treated at the blastula stage for 4 hours at 0° C.

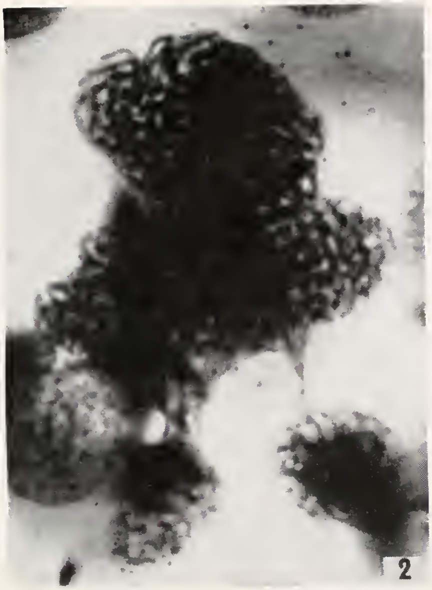
FIGURE 6. Probable tetraploid metaphase. Note the split condition of the chromosomes and the departure from the normal ring configuration of a metaphase in polar view. From a larva treated 80 minutes after insemination at 4° C. for 18 hours (1942 material).

FIGURE 7. Pentaploid metaphase. One sector of the mitosis appears to be on a different level than the remainder, and is therefore out of focus in the photomicrograph. From a larva treated at the blastula stage for 1 hour at 0° C.

FIGURE 8. Hypodiploid metaphase. Thirteen chromosomes were clearly countable, and one or two others could not be traced accurately enough to be included in the count. An adjacent metaphase (not shown in the photomicrograph) is clearly hyperdiploid, with more than 22 chromosomes present. The two figures are apparently in separate cells, although the cell boundaries are not visible. From a larva treated at the tail-bud stage for 4 hours at 0° C.

FIGURE 9. "Arrested" configuration. It is not clear whether this is an atypical telophase or a compound metaphase. Two centers of orientation are present; the chromosomes are beginning to lose their identity and to fuse with one another. From a larva treated at the gastrula stage for 2 hours at 0° C.

Plate I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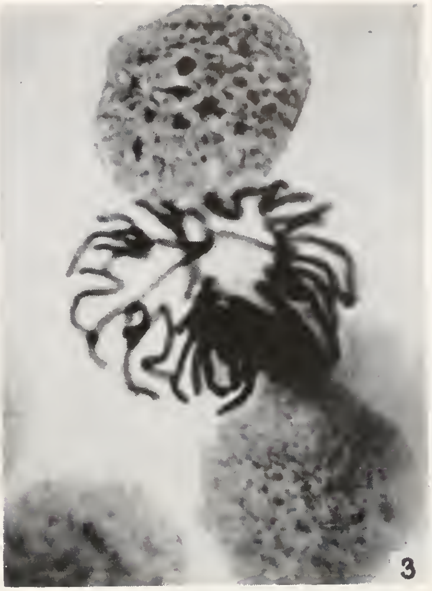
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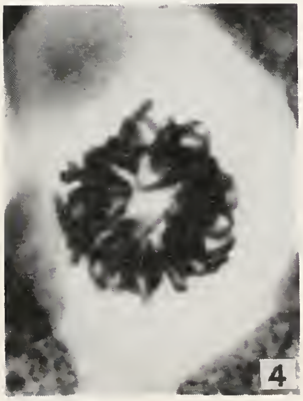
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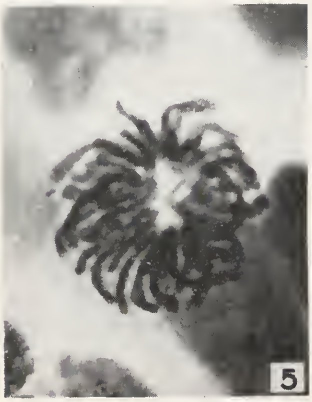
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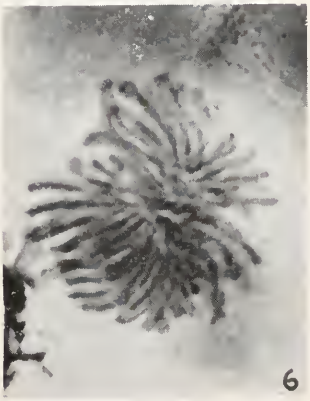
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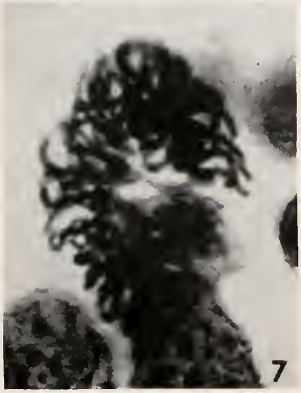
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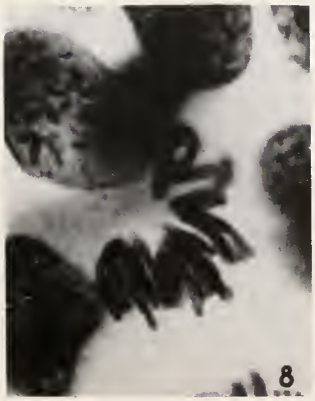
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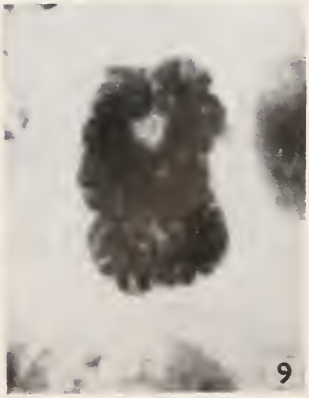
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be normal. A possible tetrapolar figure is shown in Figure 17, although the abnormal condensation of chromosomes in two of the elements makes it difficult to interpret the relationship of the four poles to one another. The orientation of all four groups toward a common center suggests that this may represent a tetrapolar anaphase. Superficially, the configuration shown in Figure 18 also resembles a tetrapolar mitosis. However, the morphology of the chromosomes in the lower pair of poles indicates that this group of chromosomes is in a different stage of anaphase than the other two.

In general, there seemed to be no correlation between the severity of the cold treatment and the nature of the mitotic abnormalities produced. The greatest number of mosaics (as shown in Table 1) was obtained by treating embryos shortly after insemination, and at the blastula and gastrula stages.

The appearance of the cold-treated larvae is often remarkably normal (Fig. 1), even though the tail-tip preparations from such animals may have quite radical anomalies of mitosis and of chromosome number. This is in accord with unpublished observations made on shipped *T. torosus* and experimentally cold-treated

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PLATE II

FIGURE 10. "Sticky" anaphase. The groups of daughter chromosomes have begun to separate, but connections persist in at least three regions. From a larva treated at the gastrula stage for 2 hours at 0° C.

FIGURE 11. "Sticky" anaphase. In this configuration only one connection persists between the daughter elements, which have completed or almost completed their movement. The dark granules are embryonic pigment. The outline of the spindle area is faintly discernible in some regions. From the same larva as the metaphase shown is Figure 7, treated at the blastula stage for 1 hour at 0° C.

FIGURE 12. Anaphase with disoriented chromosome or chromosome fragment. The "lost" element appears to be devoid of a spindle attachment; its apparent adherence to the end of one of the daughter chromosomes is unusual in this material. Granules of embryonic pigment are visible in the spindle area, which is stained the light blue characteristic at this stage. From a larva treated at the blastula stage for 4 hours at 0° C.

FIGURE 13. Anaphase or early telophase with chromosome or chromosome fragment "lost" at one pole. The figure is surrounded by a vacuolated area which appears light. Cytokinesis has begun and part of the new cell boundary is visible. From the same larva as Figure 9, treated at the gastrula stage for 2 hours at 0° C.

FIGURE 14. Telophase with two chromosomes or chromosome fragments lagging on the spindle. These appear to be homologous daughter chromosome fragments which have been lost from their respective polar groups. From a larva treated at the blastula stage for 1 hour at 0° C.

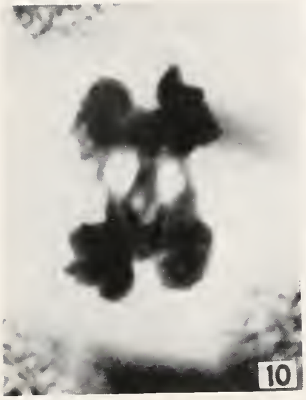
FIGURE 15. Anaphase with bent spindle. The center of orientation of one of the poles is almost at right angles to that of the other. The lightly-stained spindle area characteristic of this stage is unusually clear in this photomicrograph. From a larva treated at the tail-bud stage for 4 hours at 0° C.

FIGURE 16. Tripolar anaphase. This is an exceptionally clear example of a multipolar figure. Two of the poles appear to have fewer chromosomes than the third, but are too crowded to count. The edge of a normal prophase is visible at the right of the photograph. From the same larva as figure 12 treated at the blastula stage for 4 hours at 0° C.

FIGURE 17. Tetrapolar anaphase. The arrangement of the chromosomes in two of the four groups is irregular. All four poles appear to be oriented toward the center. From a larva treated at the blastula stage for 1 hour at 0° C.

FIGURE 18. Two adjacent anaphases with a pair of homologous daughter chromosomes lost from one of the spindles. The chromosome movements have progressed to different degrees in the two figures. From a larva treated at the tail-bud stage for 4 hours at 0° C.

Plate II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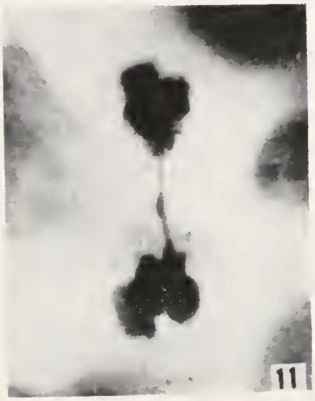
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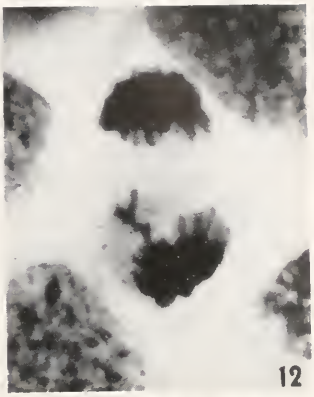
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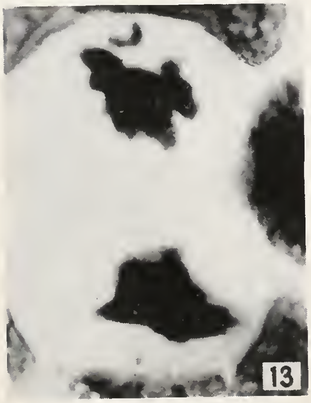
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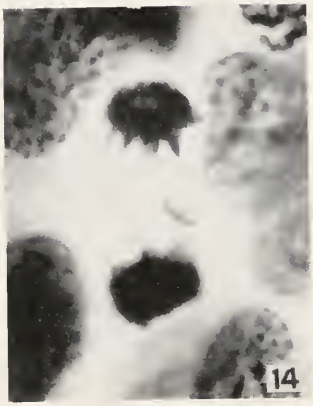
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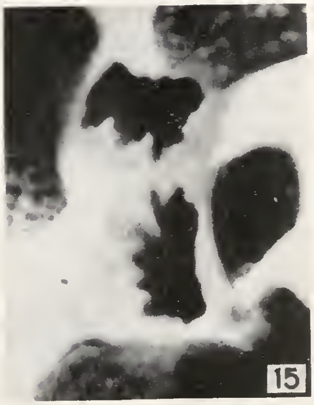
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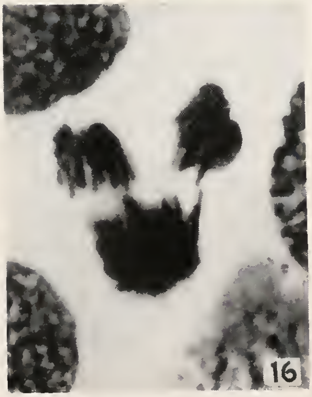
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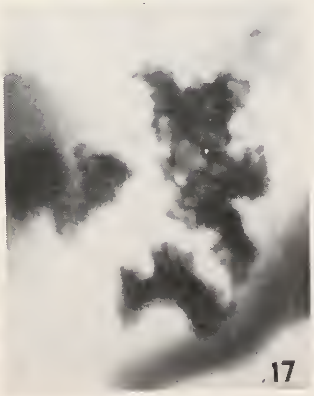
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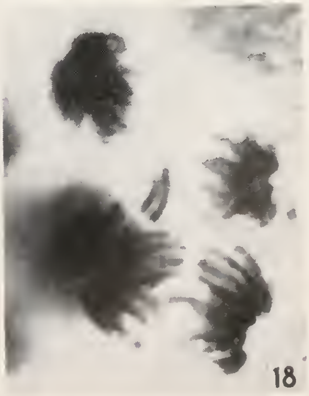
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Amblystoma punctatum larvae. In some cases, however, the atypical chromosome condition is reflected in the general morphology. Larvae have also been studied in which all the evidence indicates a normal diploid chromosome condition, even though the animal was abnormal in appearance.

DISCUSSION

A. The absence of polyploidy

Fankhauser and Griffiths (1939) devised an effective and simple method for the production of triploids in *Triturus viridescens*, based on the earlier experiments of Rostand (1934). They found that refrigeration of the eggs shortly after insemination resulted in a high percentage of triploid individuals. This effect was attributed to a suppression of the second maturation spindle which normally gives off the second polar body shortly after fertilization (Fankhauser and Griffiths, 1939). Recently, Fankhauser and Godwin (1948) have demonstrated that the most important effect of heat treatment in producing triploid *Triturus viridescens* eggs is a submergence of the mitotic figure below the egg cortex, giving two haploid egg pronuclei. Fankhauser and Watson (1942) and Briggs (1947) also have found that heat treatment shortly after insemination is effective in producing triploids. It appears that temperature shock is the important factor in all these cases, since abrupt removal of the eggs to and from the experimental temperature bath produces a considerably higher incidence of triploidy than less drastic transfers.

Possible explanations for the absence of polyploidy in a "natural population" of untreated *T. torosus* larvae have been discussed by Costello and Henley (1949). It is suggested that balanced lethal factors are often present in the diploids of this species. Triploids, having an extra dose of one or more of the lethal genes, might die early in development, due to a lethal imbalance. Presumably, a similar explanation could account for the absence of any cases of triploidy among the larvae cold-treated shortly after insemination by the Fankhauser-Griffiths method. There is, of course, no direct evidence for such a theory. Although exact records of mortality were not kept, it is perhaps significant that many of the embryos developing from *T. torosus* eggs treated by this technique died early in development. Indeed, a considerable number of experiments on single egg clutches subjected to cold shock shortly after insemination are not included in this report because the embryos died before a cytological study could be made. The possibility remains that a larger number of cases than that reported here (26) might reveal instances of triploidy after appropriate temperature shock treatment.

Fankhauser (1945) has pointed out that eggs from some *Triturus viridescens* and axolotl females are refractory to cold treatment, and produce predominantly diploid embryos. A similar difference in resistance to temperature shock may exist between species of Triturus. In any event, it is quite surprising that in a total of more than 2200 *T. torosus* larvae (normal, shipped and cold-treated) examined by Dr. Costello and the author, no completely polyploid individuals have yet been found.

B. Mosaicism

As by-products of parthenogenesis, cold, colchicine and heat experiments, and as the direct result of temperature shock later in development than that designed to produce triploidy, the production of complex mosaics has been reported in the literature over a period of years. These mosaics often possess radical mitotic abnormalities as an accompanying feature.

Parmenter (1933, 1940) found haploid/diploid, diploid/triploid and diploid/triploid/tetraploid individuals in parthenogenetic *Rana fusca* and *R. pipiens* larvae.

In a group of 100 refrigerated *Triturus viridescens* eggs, Griffiths (1941) found one haploid/triploid and five haploid/diploid larvae. Costello (1942) reported two haploid/diploid mosaics in a total of 100 larvae raised from cold-treated *Triturus similans* (granulosus) eggs. Barber and Callan (1943) studied tail-tips of cold- and colchicine-treated *Triton vulgaris* larvae, and described tetraploid mitoses resulting from these treatments. Böök (1943) chilled *Triton taeniatus* eggs later than half an hour after fertilization but before the first cleavage. The duration of treatment is not clearly specified, but appears to have been for periods of 6 to 15 hours. He reports a high mortality in the experimental eggs, only six of sixty-two cases surviving long enough for cytological studies to be made. All six survivors were complex mosaics of various types. In another series of experiments Böök (1945) treated *Triton taeniatus* and *Triton cristatus* eggs 4 to 15 hours at 2° C., more than thirty minutes after insemination. Thirteen embryos of the sixty-four treated survived to the neurula stage, at which time they were examined cytologically. Six of these thirteen cases were complex mosaics. A great variation in nuclear size and a number of mitotic abnormalities were also noted.

Briggs (1947) applied the heat-treatment technique of Fankhauser and Watson (1942) to anuran material. He treated 347 *Rana pipiens* eggs at various intervals after insemination, at temperatures of 35° and 37.5° C. for 4 to 7.5 minutes. Briggs found 32 "mixed" abnormal neurulae (9.2 per cent), which appear to have been haploid/diploid or haploid/triploid mosaics.

Fischberg (1947) subjected eggs of *Triton alpestris* to cold shock within 30 minutes after insemination. Among 553 larvae studied cytologically by the tail-tip method, he observed 27 haploid/diploid, 20 haploid/triploid, 5 diploid/triploid, and 1 diploid/triploid/tetraploid mosaic individuals. Eight larvae were non-mosaic aneuploids, and the remainder were euploid (haploid, diploid, triploid and tetraploid).

In general, the results of a study of the cold-treated *Triturus torosus* embryos are quite comparable to those discussed above. The incidence of chromosomal mosaicism was significantly higher than in the control larvae, although it was not as high as that (43 per cent for a sample of 126 tips) reported for *T. torosus* embryos subjected to shipment (Costello and Henley, 1947, 1948). This is understandable in view of the fact that embryos shipped for a considerable distance in ice and water are subjected to low temperatures for several days. Most of the experimental treatments reported here were not so prolonged. A few tests were made for comparable periods, but the embryos died before they could be tail-clipped. The "D" group of larvae cold-treated in 1942 shows a remarkably high frequency of mosaicism (5 larvae of 13 treated for 18 hours, beginning 80 minutes after insemination). This is a considerably longer period of cold-treatment than the majority of the experiments reported here. The high degree of effectiveness of the cold shock in producing mosaicism in this group of eggs may be due to the fact that the treatment was operative during an especially susceptible physiological state of the zygote.

The mosaics found in the study of cold-treated embryos were much more radical than those occurring in the "natural population" studied as a control series (Costello and Henley, 1949). This is in contrast to the qualitative similarity of mitotic abnormalities in the control and experimental tips. Two of the three mosaic tail-tips observed among the controls were apparently diploid/hyperdiploid, and the third was diploid/tetraploid. One of the diploid/hyperdiploid tips was from an animal collected and raised under constant temperature conditions. The remaining two were from larvae which had been collected in very cold water. None of these three cases was as markedly atypical as the majority of instances of mosaicism among the experimental larvae. It is probable that none had been exposed to as pronounced temperature shock as the experimentally cold-treated animals. This is interpreted to indicate that there may be a direct relationship between the severity and duration of the cold shock and the degree of cytological abnormality resulting in a given individual. However, Böök (1943) expresses the opinion that no such relationship holds in Triton.

The frequency of mosaicism (especially of aneuploid constituents) may be considerably higher than the data indicate since, as was noted above, many of the metaphases are too crowded for accurate chromosome counts. Evidence for this possibility is afforded by the high incidence of variations in nuclear size and nucleolar number. Böök (1945) also reports a great variation in nuclear size in cold-treated Triton larvae. Since both of these criteria are known to be indices of heteroploidy, it seems quite likely that some of the variations found represent interphase nuclei with varying chromosome numbers.

C. Mitotic abnormalities

There have been a number of descriptions of abnormalities of spindle formation in amphibian nuclei, following irradiation, cold and colchicine treatments. An early report was that of Alberti and Politzer (1924a, 1924b) who observed the effects of x-rays on embryos of *Salamandra maculosa* eight days after insemination. The corneal epithelium of these larvae was studied at varying intervals following irradiation, and a variety of anomalies of mitosis, similar to those reported in the present study, was described. Böök (1943) treated neurulae at 2° C. for 8 hours, and examined the embryos cytologically five hours after the end of the treatment. He observed a tripolar anaphase and other abnormalities of spindle formation. Barber and Callan (1943) described a number of types of mitotic abnormality observed in tail-tips from cold- and colchicine-treated *Triton taeniatus* larvae. Many of these abnormalities were very similar to the ones described in the present study (e.g., "sticking" anaphases).

In the cold-treated *T. torosus* material, the frequency of various types of mitotic abnormality is higher in the experimental tips than in the control. Furthermore, it is appreciably higher in the regenerated experimental tips than in the "first" tips. There is a possibility that cold-treatment has delayed effects on mitosis which are expressed only after the stimulus of clipping has occurred. If this is so, such delayed effects must be operative for a relatively short time, since the incidence of mitotic abnormality in third clippings is apparently not as high as in the second clippings. This has been found to be the case in tail-tips from shipped embryos, although no data are available for experimentally cold-treated individuals. Briggs (1947) has pointed out that in heat treated *Rana pipiens* larvae, mitotic abnormalities do not show up until the blastula and gastrula stages. A comparable retarded expression of effects might be involved in these cold-treated larvae. There is also the possibility that the higher incidence in "second" tips may be explained by the fact that such preparations usually have more mitotic figures than either "first" or "third" tips, and are generally more favorable for cytological study.

The frequent occurrence of mitotic abnormalities is of considerable interest when compared with that found in tail-tips from a "natural population," although it is lower than the incidence in tail-tips from shipped larvae (unpublished data). In general, the difference is quantitative rather than qualitative, since the types of abnormality observed are comparable to those found in both normal and shipped larvae. The main exception to this statement is found in the occurrence of multipolar spindles in the experimentally cold-treated and in the shipped larvae. No such configurations were observed in control tips; they may be the result of quite radical and prolonged temperature shock, as well as of other factors.

D. The relationship between morphological and cytological characteristics

The normal appearance of many of the cold-treated larvae affords additional evidence for the idea expressed by Costello and Henley (1949) that the general morphology and pigment pattern of a given larva are not necessarily valid criteria of its chromosome condition. Presumably, there is a fairly close correlation between the size of the pigment cells and the number of chromosome sets present in euploid individuals, but this assumption cannot be taken to apply too rigidly. Thus, Fankhauser (1945) points out that such a criterion is not always reliable in the case of axolotl larvae, where the size of the pigment cells in triploids often is not clearly distinguishable from that in diploids. In view of these findings, morphological characteristics must be used with caution as a criterion of heteroploidy. The fact that *T. torosus* larvae abnormal in general appearance may be cytologically normal confirms this belief.

E. General effects of low temperature

It is interesting to speculate on the possible reasons for the special susceptibility of anaphases and telophases in *T. torosus* to the effects of cold. Costello and Henley (1949) have suggested that the lagging and lost chromosomes and chromosome fragments noted may be due to the fact that those chromosomes lacking kinetochores would be left behind in the migration of the daughter elements to opposite poles. Since no such migration has yet occurred at the metaphase, the loss of kinetochores would pass unnoticed unless the chromosomes failed to become located on the equatorial plate. It is also possible that there is a decrease in the adhesive qualities of the chromosomes or of the spindle fibres during anaphase and telophase. This would result in the higher incidence of lagging or lost elements reported at these stages.

Böök (1943, 1945) attributes the effects of cold to a partial or complete paralysis of the spindle apparatus. This might be especially conspicuous at anaphase and telophase, interfering with the normal movements of the chromosomes. He interprets his observed cases of atypical metaphase configurations as being due to the loss of ability of the centromeres to orient themselves properly. Such an explanation of the facts seems rather vague, since it does not account for the actual cause or manner of loss of the capacity of the centromeres for orientation.

It is difficult to ascribe the cytological abnormalities obtained after cold treatment to any specific effect of the low temperature. Böök (1945) interprets the origin of mosaics as a result of the omission of nuclear and cell division after cold treatment, with the retention of the division rhythm of centrosomes and chromosomes. Fischberg (1947) suggests three possible modes of origin of the aneuploid constituents of mosaics: (1) loss of single chromosomes at anaphase; (2) failure of chromosome division; (3) disorderly division of chromosomes in multipolar figures. From the evidence obtained in the present study of cold-treated *T. torosus* larvae, it seems possible that any or all of these factors may be operative in the production of the observed abnormalities. Since the loss of single chromosomes at anaphase appears to occur quite frequently, it may be that this is an especially important factor, as pointed out by Costello and Henley (1949). "Physiological polyspermy" normally occurs in the fertilization of salamander eggs; thus, there is also the possibility that low temperatures affect the supernumerary sperm nuclei so that they contribute to the formation of mosaic areas instead of degenerating as is usually the case.

In general, the evidence from this study indicates that low temperatures may result in the production of chromosomal mosaics and abnormalities of mitosis, similar to those observed in tail-tips from the larvae shipped in ice and water. The relative roles of anoxia, agitation and other factors in the shipped material, however, remain obscure.

SUMMARY

1. A study of the incidence of chromosomal mosaicism and abnormalities of mitosis has been made, utilizing tail-tip preparations made from larvae of *Triturus torosus* subjected early in development to low temperature.

2. Three hundred-ninety two "first" tail-tips and 294 regenerated "second" tail-tips have been studied cytologically from preparations made in 1948. An additional 27 preparations made in 1942 have also been studied. Similar observations were made in 1948 on tail-tips from 582 control *T. torosus* larvae.

3. The experimental larvae were exposed to low temperatures (0 to 8° C.) for varying periods of time (45 minutes to 23 hours) at stages of development from 5 minutes after insemination, through blastula, gastrula, neurula and tail-bud stages.

4. No haploid or polyploid individuals were observed, in either the control or experimental groups.

5. Of the 392 "first" tips, 153 (39 per cent) were normal diploids; 156 (40 per cent) had nuclei of variable size or variations in nucleolar number; 79 (20 per cent) had abnormal mitoses; and 4 (1 per cent) were chromosomal mosaics. Of the 294 "second" tips, 71 (24 per cent) were normal diploids; 107 (36 per cent) had nuclei of variable size or variations in nucleolar number; 102 (35 per cent) had abnormal mitoses; and 14 (5 per cent) were chromosomal mosaics. Of the 27 tips prepared in 1942 from cold-treated animals, 5 were clearly diploid, 11 had nu-

clei of variable size or variations in nucleolar number, 6 had abnormal mitoses, and 5 were chromosomal mosaics. In general, the incidence of all types of cytological abnormality was higher in the regenerated than in the "first" tips. This incidence of abnormality is significantly higher than that found in the controls.

6. The types of mosaics found included: hypodiploid/diploid/hyperdiploid; diploid/hyperdiploid; diploid/triploid; diploid/triploid/tetraploid; diploid/triploid/pentaploid; diploid/tetraploid.

7. A wide variety of abnormalities of mitosis was noted, including: "sticky" anaphases and telophases; anaphases and telophases with lagging or lost chromosomes or chromosome fragments; anaphases and telophases with bent spindles; multipolar spindles. Masses of relatively uncondensed chromatin and interphase nuclei with persistent connections between the daughter components were also present.

8. The evidence indicates that *T. torosus* larvae exposed early in development to low temperatures may suffer quite radical changes in chromosome number in some body cells, as well as less extreme abnormalities of mitosis. This is in accord with the findings of other workers for other species, and offers a clue to the possible mode of origin of the cytological abnormalities observed in *T. torosus* embryos subjected to shipment in an ice-and-water mixture.

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CARBONIC ANHYDRASE AND GROWTH IN THE OYSTER AND BUSYCON [1, 2]

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The enzyme carbonic anhydrase is present in the shell-forming mantle tissue of a large number of mollusks (van Goor, 1948; Freeman and Wilbur, 1948). During the course of an investigation of the possible importance of this enzyme in the deposition of carbonate in shell formation it was noted that the carbonic anhydrase concentration of the conch *Busycon carica* was considerably less in larvae just prior to shell formation than in those which had recently acquired a shell. This suggested that the enzyme content may increase with development and raised the question as to the constancy of the enzyme content of the mantle tissue with growth of the adult. These aspects of carbonic anhydrase activity have been examined in the present study. The alteration of enzyme activity during growth has been investigated on the whole larvae of *Busycon carica*. For a study of enzyme activity during growth of adult mantle the oyster *Ostrea virginica* has been used. The results indicate that the carbonic anhydrase activity varies in a regular manner in these forms both during development and with growth of the mantle of the adult.

METHODS

Ostrea mantle tissue and whole Busycon larvae taken from egg cases were ground and usually frozen. The tissue was then extracted in 3 to 9 times its weight of distilled water for two or more hours. Oyster sperm and eggs were washed in sea water by centrifugation before extraction. Tissue samples to be compared for enzyme activity were prepared at the same time and received identical treatment. The extracted material was centrifuged briefly to remove tissue and shell fragments and the carbonic anhydrase activity of the supernate was measured colorimetrically using a veronal buffer (Wilbur and Anderson, 1948). Sulfanilamide, a powerful inhibitor of mammalian carbonic anhydrase (Mann and Keilin, 1940), was found to inhibit the enzyme in Busycon and Ostrea. Accordingly, an aliquot of an experimental sample to which sufficient sulfanilamide was added served as a very satisfactory control. By comparing the rate of enzyme action in the presence and absence of sulfanilamide the action of tissue substances which affect the pH and buffer capacity of the medium is cancelled. The enzyme activity was calculated

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[3] Atomic Energy Commission Fellow.

from rates of the catalyzed and uncatalyzed reaction and expressed in units following the method of Main and Locke (1941).

Unfortunately no single tissue unit will serve satisfactorily as a basis for comparison for all the tissues employed. For mantle tissue, wet weight and dry weight have been used. For whole Busycon larvae, both with and without shell, the enzyme activity is expressed as units per individual and also per unit volume as determined by centrifuging thoroughly macerated larvae at ca. 825 × g. for 5 minutes. The latter method was also used for Ostrea eggs and sperm.

RESULTS

Busycon larvae

The eggs of Busycon are laid in a string of flattened, disk-like cases which commonly contain two to three dozen or more individuals. Since the formation of the string of cases continues for some period, one has available for study in a single

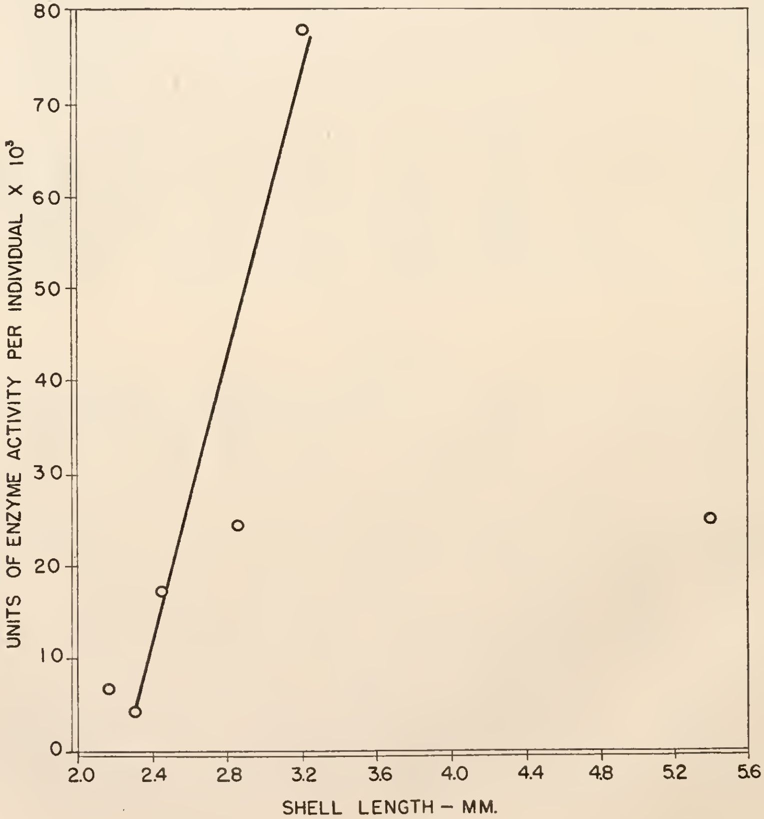

FIGURE 1. Carbonic anhydrase activity of Busycon larvae. Approximately 300 larvae of similar size from a single egg string were extracted for the determination of each point. Several egg strings were used. A similar relationship is obtained on plotting enzyme activity vs. unit volume.

string a series of developmental stages both with and without shell. The carbonic anhydrase activity of naked larvae and those with shell from such a string was measured on a sample of about 300 individuals of each type. The enzyme activity per individual was found to be 0.0049 units for those without shell and 0.23 units for those with shell, or an increase of 47 fold. The jelly within the egg cases was without enzyme activity.

The difference in enzyme activity in the naked and shelled stages suggested an examination of possible variation in enzyme activity with development in a series in which all larvae were depositing shell. Lots of 150 to 300 individuals in a desired size range were collected. A sample of 16 to 32 individuals was taken for an estimate of the average length, and the entire lot was then used for the measurement of enzyme activity. When the enzyme activity per individual is plotted against average length (Fig. 1) it is seen that the activity increases very markedly during the early stages of shell deposition. The activity of an individual 3.2 mm. in length may be nearly 20 times that of one 2.2 mm. in length. Clearly the increase per unit of tissue is also very great indeed. This may be shown by expressing the activity in terms of unit volume as determined by centrifuging macerated larvae, even though a large fraction of the total is shell. Whether enzyme activity falls off for individuals larger than 3.2 mm. (Fig. 1) or whether the value for the 5.4 mm. stage is aberrant is not known.

The changes in carbonic anhydrase activity with growth in Busycon reported here represent the enzyme activity of the whole larva. If the increase in enzyme activity found in Busycon is to be correlated specifically with shell deposition, the mantle tissue should also show the increase, since this tissue is responsible for shell formation. Unfortunately the measurement of enzyme activity of the mantle of individuals of 2 to 6 mm. in length will have to await the development of suitable micro methods.[4] The relation between size and enzyme activity of the adult mantle has, however, been investigated in Busycon and also in Ostrea.

Adult Busycon

Individuals ranging in length from 5 to 20 cm. and weighing from 8.9 to 587 gms. were used. Because of the difficulty of grinding mantle tissue, pieces of mantle were sliced very thin with a razor while frozen and extracted at low temperature for 48 hours. In a single series examined no consistent relationship between size and enzyme activity was found, although there was some slight suggestion that the mantles of the largest individuals had a somewhat lower enzyme content than the smallest individuals on a wet weight basis. Further study on this species would be desirable.

Adult Ostrea

In the adult oyster a definite relation was found between size and enzyme activity. The activity per unit dry weight or wet weight of mantle was in general inversely proportional to the length of the shell (Fig. 2). The activity of the smallest individuals may be several times that of the largest in any one collection.

The curves for oysters from two beds a few hundred yards apart are character-

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[4] A micro method for carbonic anhydrase is being devised for such quantities of tissue.

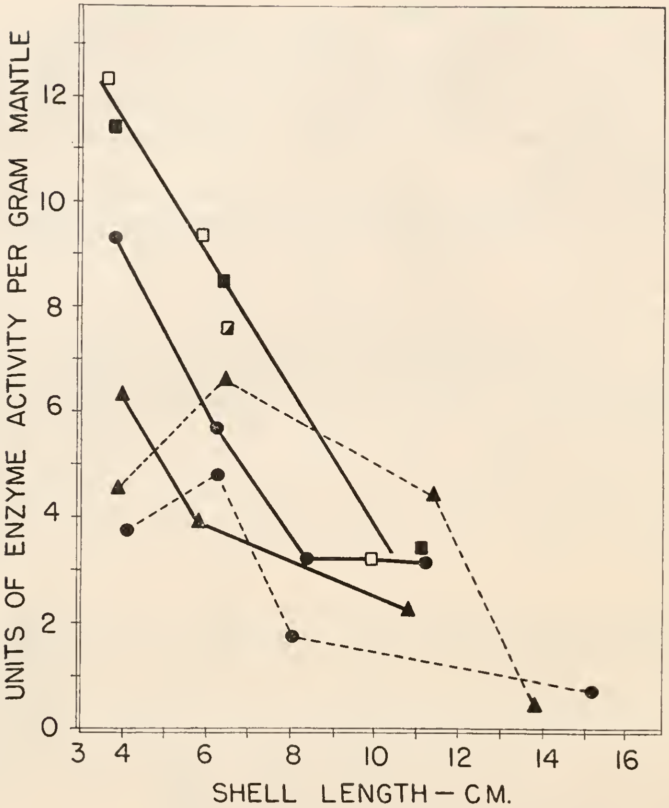

FIGURE 2. Carbonic anhydrase activity of oyster mantle. Enzyme activity is expressed per gm. wet weight. Dry weight gives a similar relationship. The shell length represents average measurements on the shorter shell. Data plotted with solid lines (————) are for oysters taken from a bed located on a sandy beach exposed to relatively rapid water movement. Data plotted with dashed lines (- - - -) are for oysters taken from an intertidal bed in muck containing fuel oil from ships and in a location not exposed to fast currents.

□ July 12, 1948.
■ July 12, 1948. Oysters kept in air 6½–7½ hours.
◪ July 12, 1948. Oysters kept in air 6½–7½ hours, then returned to water for 13 hours.
▲ October 31, 1948.
● March 5–6, 1949.

istically distinct both in their form and with respect to seasonal differences.[5] The data indicated in solid lines in Figure 2 were obtained from oysters collected between low and high tide levels from a sand beach exposed to relatively rapid water movement. Dashed curves (Fig. 2) are for oysters growing between tide levels in muck containing fuel oil from ships and in a location which was not subjected to fast currents.

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[5] Dr. A. F. Chestnut of the Institute of Fisheries Research of the University of North Carolina, Morehead City, N. C., found active deposition of shell by oysters in this region throughout this period.

The enzyme activity of oysters exposed to air for 6½ to 7½ hours (Fig. 2, ■) was the same as that for oysters taken directly from sea water (Fig. 2, □).

By extrapolation of the curves in Figure 2 one might predict the size of oyster which would be completely deficient in carbonic anhydrase. Interestingly enough, oysters of this size have not been found in the beds from which the experimental material was collected.

Discussion

The presence of carbonic anhydrase in a wide variety of tissues of animals belonging to several phyla suggests that the enzyme plays a general role in the handling of metabolic carbon dioxide (van Goor, 1948). Such a role seems likely in mollusks as well, for the enzyme is present in several tissues. An analysis of washed eggs and sperm of the oyster showed that both gametes also contain carbonic anhydrase. The eggs had an activity of 3.6 units and the sperm 3.4 units per ml. of packed cells. Here too a general rather than a specific function appears probable. With differentiation the concentration of the enzyme may increase as described for Busycon larvae and has also been found in vertebrate tissues (Van Goor, 1948). Whether the marked increase in carbonic anhydrase bears a general relation to development and differentiation or has a more specific function in the rapid deposition of shell which occurs at this time, is not known.

In contrast to Busycon larvae, enzyme activity of the adult oyster mantle was found to decrease with increasing size. The possible physiological significance of this relationship for shell deposition obviously depends upon whether the enzyme activity becomes a limiting factor in the processes concerned with shell formation. If the enzyme is in excess, as it is in mammalian erythrocytes, a reduction may be of no consequence. An evaluation of the part played by carbonic anhydrase is being approached by studying the influence of enzyme inhibitors on the rate of shell growth as indicated by the deposition of radioisotopes.

The change in carbonic anhydrase content of the oyster mantle with age does not in itself reflect a decreased ability to synthesize the enzyme but may be due to a relative increase in cells or material not directly involved with carbonic anhydrase. Speculation in this regard appears unprofitable until such time as a histochemical technique for the enzyme can be applied to the mantle tissue.

The variation of carbonic anhydrase activity of the oyster mantle with size, habitat, and with the season suggests that these factors should be considered in comparing carbonic anhydrase activity of species of mollusks and perhaps other invertebrates.

Summary

1. The carbonic anhydrase activity of *Busycon carica* was found to increase markedly during development in the egg case. The enzyme activity of whole larvae during the early stages of shell formation may be more than 40 times that of larvae just prior to shell deposition. Larvae with shell may show an increase of nearly 20 fold in enzyme activity in growing from a length of 2.2 mm. to 3.2 mm.

2. In the mantle of the adult oyster *Ostrea virginica* the carbonic anhydrase activity was in general inversely proportional to the length of the shell. The activity per unit weight of mantle of small oysters may be several times that of large

oysters growing in the same location. Curves relating enzyme activity of the mantle to the size of the oyster were distinctly different for oysters growing in different habitats.

3. Carbonic anhydrase is present in both gametes of the oyster, the concentration per unit volume being of the same order in both.

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A PRELIMINARY HISTOLOGICAL STUDY OF THE INSEMINATION REACTION IN DROSOPHILA GIBBEROSA [1]

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The insemination reaction was first described by Patterson (1946) in Drosophila, and has been subsequently studied in numerous species of the genus by Wheeler (1947). The reaction occurs strongly, weakly or not at all depending on the species being studied. When present as a strong reaction (as in *Drosophila gibberosa*) it consists of a swelling of the vagina to three to four times the normal size following mating. This swelling is brought about by the secretion of fluid from the vaginal epithelium in response to some stimulus supplied by the semen; in at least some respects it resembles an immunological reaction. Commonly, especially in reactions resulting from the mating of different species, a more or less opaque mass develops in the lumen of the vagina; this is termed the "reaction mass."

Previous work has been limited to the observation of gross dissections and the determination of genetical results. The present study was initiated to extend the study to histological structure and to determine the specificity of the reaction, and perhaps the steps involved, by the artificial insemination of materials other than semen.

Materials and Methods

Drosophila gibberosa was selected because of its large size, availability and ready rearing on standard media. Since adults of this species do not mate until nine to fourteen days after emergence, males and females were kept separately in humid milk bottles and fed bananas or a yeast-honey mixture during this period.

Mating can be obtained with single females and several males in separate containers, but for the purposes of this work mass methods were more satisfactory. Ten to twenty females and a similar number of males were placed in glass containers of approximately one pint capacity. The containers were lined with wet filter paper to maintain a high humidity. Reasonable numbers of matings occurred within 30 minutes. Mating pairs were isolated by inverting small vials over them, the vials being numbered corresponding to notes kept on each pair.

The abdomens of mated females were cut off and fixed in Brazil's (alcoholic Bouin's) fluid at 0, 5, 15, 30, 60, 120, 180, 240, 300, and 360 minutes after the beginning of mating. In some tests intervals as long as 11 hours were used. They

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The writer wishes to express her sincere appreciation to Dr. A. G. Richards of Division of Entomology and Economic Zoology, University of Minnesota for suggesting the problem, for supervision during the course of this study and for reading of the manuscript.

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were then washed, dehydrated, sectioned in paraffin (Tissuemat), and stained with Delafield's haematoxylin and eosin or acid fuchsin and congo red by routine methods.

For artificial insemination the desired organs were dissected free in saline,[3] cleaned of adhering tracheae and other tissues, and ground into a brei in saline using a small mortar and pestle. Capillary glass tubes sealed onto ordinary syringe needles were drawn to desired fineness and used for injecting into the vaginae. Etherized females were placed ventral side up on the stage of a binocular microscope, held gently with forceps, and the needle (preferably held in an adjustable mechanical stage) manipulated through the gonopore into the vagina. In the present preliminary work, the injections were not quantitative but were roughly the same volume. Artificially inseminated females and ones injected with non-semen experimental fluids were prepared for histological examination in the same manner as the normally mated females.

Fluids injected into the lumen of the vagina artificially include:

1. Yeager's cockroach saline;[3] this has a tonicity equal to a 1.3 per cent solution of NaCl.
2. 2 per cent NaCl solution.
3. 2 per cent gelatine in cockroach saline solution.
4. brei of testes in saline solution.
5. brei of male accessory glands in saline solution.
6. brei of male accessory glands plus testes in saline solution.
7. brei of entire male fly in saline solution.

The volume of epidermal cells of the vagina was estimated by making camera lucida drawings of each section on calibrated graph paper, adding up the number of squares occupied by the drawings (estimating partial ones), and multiplying by the thickness of the sections. Probably a rather high percentage of error is involved in these estimates, but since the differences to be discussed are differences of several magnitudes the errors would not seem serious. It should be remembered, however, that all areas and volumes represent measurements on sectioned material; the figures are doubtless smaller than life size but should be accurate for relative sizes.

Results

1. Macroscopic features of vaginae of virgin females: The term vagina in insects has been variously used. Snodgrass (1935) regarded the vagina as that part of the female reproductive tract which extends posteriorly from the base of the median oviduct to the ovipositor. In dipterous insects, Pantel (1910) referred to it as the uterus (Townsend, 1911). Nonidez (1920) divided this region into two parts, a uterus proper and a vagina. For Drosophila, Patterson (1947) followed the terminology of Snodgrass but added the term "vaginal pouch" for the portion of vagina which extends antero-ventrally. During the course of insemination reaction, the wall of the vaginal pouch is capable of great distention, the wall of the vagina remains unchanged. For descriptive convenience, the terminology of Patterson was adopted in the present study.

[3] Yeager's cockroach saline: NaCl, 10.93 grams; KCl, 1.57 grams; $CaCl_2$, 0.85 gram; $MgCl_2$, 0.17 gram; $NaHCO_3$, 0.17 gram per liter of double distilled water.

In *Drosophila gibberosa* the vagina is a whitish pear-shaped body, situated at the posterior end of the abdominal cavity just beneath the rectum. The vaginal pouch is about one-third as large as the vagina and is situated antero-ventrally to the origin of the parovariae and ventral receptacles. Both vagina and vaginal pouch are provided with a well-developed muscular wall and are without significant demarcation externally. The gonopore is enclosed between two valves of the ovipositor which are located at the posterior end of the abdomen externally.

2. Microscopic structure of vaginae of virgin females: The vaginal wall is composed of three distinct layers. The innermost layer is a transparent thin non-cellular cuticular intima which is more prominent in the vagina than in the vaginal pouch. Apparently it was the endocuticular portion of this that was called a separate fibrous layer by Nonidez (1920) in *Drosophila melanogaster*. Second, there is a single layer of cuboidal epithelial cells, the nuclei of which are more or less oval, located usually in the middle of the cells and generally with distinct chromatin threads and one or two nuceoli. The epithelial layer is much thicker than the intima: it is slightly thinner on the wall of vaginal pouch and with more scattered and sparse cells as compared with aggregated and dense ones of the vagina. The epithelium and the intima are thrown deeply into many folds within the lumen, especially so on the wall of the vaginal pouch; thus the layers of cells appear to be superimposed on others in the section. Third, externally the vagina is lined by a thick muscular layer which is much thinner and sparser on the wall of the vaginal pouch. Presumably a basement membrane and some other slight connective tissue elements are present but these are not sharply demarcated. The volume of epithelium measured from three sets of serial sections averages 1,270,000 cubic microns.

3. Macroscopic features of vaginae of mated females: Immediately after mating the vaginal pouch begins to increase in size. In some cases the vaginal pouch reaches its greatest distention about two and one half hours after mating; in other cases four hours is required to attain the greatest distention. The size at greatest distention is about four or five times as large as the normal one. The actual size attained is the sum of (1) initial size, (2) amount of semen, (3) amount of material added to semen, and (4) any changes that occur in the vaginal wall. These will be discussed later.

The wall of the vaginal pouch becomes very thin and transparent. The vagina only slightly increases in size. Within the vaginal pouch an opaque reaction mass can be seen under the microscope. The vaginal pouch begins to contract after reaching its greatest distention and the opaque reaction mass also gradually decreases in size. It returns to original size and form about five hours after mating.

4. Histology of vaginae of mated females: As the wall of vaginal pouch is gradually distended after mating, its epithelial cells become flatter and flatter and the folds within the lumen finally disappear. The intima also becomes thinner. The muscular layer becomes very thin and the muscles widely separated. The vagina is less distended; its epithelial cells remain cuboidal. As a rule the nuclei of epithelium of reacting vaginae are generally more dense as compared with those of the virgin female and without the diffuse appearance of chromatin threads; the nuclei are frequently elongated and generally located at the base of the cells. The cell volume increases to about four times the normal at its greatest distention; it averages 4,800,000 cubic microns from the measurement of three sets of serial sections of vaginae.

After reaching its greatest distention, the vagina gradually returns to its normal histological structure and about five hours after mating there is no significant histological difference from that of the virgin female. The reaction mass is composed of spermatozoa and substance of acidophilic staining property; its increase and decrease of size keeps well in pace with the distention and contraction of the vagina. The entering of spermatozoa into the ventral receptacle can be traced in the sections.

5. Macroscopic features of vaginae of artificially inseminated females: Testes brei in saline, male accessory gland brei in saline, testes plus male accessory gland brei in saline, entire male fly brei in saline, cockroach saline, 2 per cent NaCl and 2 per cent gelatin in saline were used for artificial insemination. The results are different for different injection materials.

After the injection of testes brei the vaginae undergo macroscopic changes similar to those of the normal mated female. The same feature is found in the vaginae of testes plus male accessory gland injections but the distention is of lesser degree. Injection of saline also brings about the same amount of distention. The injection of male accessory gland brei, entire male fly brei, 2 per cent gelatin and 2 per cent NaCl may bring about an unsignificant increase of vaginal size by mechanical distention of fluid injected into the lumen, but the vaginal pouch does not thin out as in other experiments.

6. Histology of vaginae of artificially inseminated females: The vagina of testes brei injections undergoes a histological change similar to that of the mated female as far as the epithelium, intima, muscles and reaction mass are concerned. The volume of epithelial cells also increases; an average of measurement of two vaginae has the same figure as that of the mated female, that is 4,800,000 cubic microns.

The vagina of testes plus male accessory gland brei injections undergoes a similar histological change to that of the previous case, but the cells on the wall of the vaginal pouch do not thin out so extensively. The reaction mass is somewhat smaller in size, but the significance of this is open to some question, as will be discussed later.

The vaginae of male accessory gland brei injection, whole male brei injection and 2 per cent gelatin injection did not show any significant histological change.

The vaginae injected with saline (which apparently is hypotonic) undergo a peculiar histological change, in which the vaginal pouches thin out moderately with the outer muscle layer retained. Unlike the nuclei of the vaginal epithelium of the mated female, the nuclei are generally of diffuse appearance. Th cytoplasm is generally more lightly stained by eosin as compared with that of the virgin or mated female. Although the vaginae are distended moderately there is no reaction mass found in the lumen. The volume of cells also increases, but to a lesser degree, average of measurement of two sets of serial sections is 3,420,000 cubic microns.

Contrary to the results from saline injection, the vaginae after injection of 2 per cent NaCl does not exhibit significant distention. The cytoplasm of the vaginal epithelium is generally heavily stained by eosin and significant shrinkage is shown at certain areas. The nuclei are also slightly shrunken. The cell volume does not increase, an average of measurement of two sets of serial sections is 1,311,000 cubic microns which is close to the figure of virgin female.

The reaction of vaginae to different kinds of inseminations is summarized as follows:

Kinds of insemination	Increase of vaginal size	Increase of volume of vaginal epithelium	Reaction mass plus intense staining
Normal mating	++	++	+
Testes in saline	++	++	+
Testes plus accessory glands in saline	++	++	+
Saline (hypotonic?)	+	+	–
Male accessory glands in saline	+	0	0
Entire male fly in saline	+	0	0
2% gelatin in saline	+	0	0
2% NaCl (hypertonic?)	+	0	0

The volume of epithelial cells of vaginae of virgin females, mated females and females artificially inseminated with testes brei, saline and 2 per cent NaCl were measured. As stated under the MATERIALS AND METHODS, these figures are relative rather than absolute because they were made on sectioned material which unquestionably shrunk somewhat during the course of preparation. The average figures for the volume of cells are tabulated as follows:

Type of female	The volume of vaginal epithelium (in cubic microns)	Average (in cubic microns)
Virgin female	1,568,116 1,285,274 1,046,470	1,270,000
Mated female	4,056,381 4,895,334 5,448,870	4,800,000
Female of testes injection	3,502,842 6,097,545	4,800,000
Female of saline injection	3,433,653 3,596,533 3,234,726	3,420,000
Female of 2% NaCl injection	1,407,400 1,215,358	1,311,000

DISCUSSION

Patterson (1946) described the presence or absence of the insemination reaction in thirty-five species of Drosophila. In 1947 he made an experiment of homogamic matings of eight species and twenty-six crosses of the species of the genus Drosophila. He found that in homogamic matings it takes five to ten minutes after mating for the sperm to move forward to the point of origin of the ventral receptacle and several hours to enter the ventral receptacle. In heterogamic matings similar movement of sperm take about forty-five minutes and the spermatozoa frequently form a tangled mass about the opening to the ventral receptacle. He pointed out that this has the effect of retarding or inhibiting the entrance of sperm into the lumen of the receptacle. In many interspecific crosses, the sperm never fertilize the eggs due to the deleterious effect of the insemination reaction and the resulting reaction mass. The egg may disintegrate in the cavity of the vagina.

Patterson (1946) inferred that the insemination reaction may have three possible functions: It may have the effect of (1) preparing the reproductive tract for the fertilization mechanism, especially in the case of homogamic matings: and it may have a direct bearing on the problem of speciation both (2) by deterring genic interchange and (3) by limiting the number of matings an individual female may have.

As regards the induction of an insemination reaction among homogamic matings of *Drosophila aldrichi*, Patterson (1947) found in one case that the vagina had already reached its maximum reaction, although the female was dissected before the termination of mating. Yet, in the distended vagina or ventral receptacle there was not a single spermatozoon. From this, he assumed that the spermatozoa are not the active agent to bring forth the insemination reaction. He undertook a convincing experiment to support his assumption. In the backcross of the sterile F_1 male from the *mulleri/mojavensis* with *mulleri* females, the vagina is slightly distended. Since the semen of this F_1 hybrid male is sperm free, the result demonstrates conclusively that the presence of spermatozoa are not necessary for the induction of the insemination reaction.

The knowledge about the exact composition of the semen of Drosophila is meager. According to Nonidez (1920) the accessory glands secrete a sticky fluid with floating refractive granules and this fluid becomes mixed with the spermatozoa from the testes to form the ejaculate. However, although the spermatozoa were found not necessary to induce reaction, at least in the above experiment, Patterson does not conclude that the secretion of the male accessory gland has such a function. He stated that the semen of F_1 hybrid of *mulleri/mojavensis* is sperm-free but does not exclude the possibility that degenerative products from the abnormal testes may constitute a part of the semen. This must await fractionation of semen into its components. Patterson points out superficial similarities to immunochemical reactions but direct evidence is lacking.

Wheeler (1947) classified seventy-eight species belonging to the genera Chymomyza, Scaptomyza and Drosophila of the family *Drosophilidae* into three classes according to the degree of insemination reaction in homogamic matings. Class 1 is composed of those forms in which no reaction is apparent; he lists seventeen species including the common *D. melanogaster*. Class 2 contains those in which a slight or moderate reaction develops; he lists forty-one species. Class 3 contains those in which there is a considerable enlargement of the vagina and formation of a dense opaque reaction mass; he lists twenty species. He also considered the problem of evolution of Drosophila from the viewpoint of insemination and the possible function of an insemination reaction in homogamic matings. As indicated by Patterson, it may have the effect of preparing the reproductive tract for the fertilization mechanism in homogamic matings. It may be correlated with the slower movement of the spermatozoa into the ventral receptacle of the immobilization of the sperm possibly due to the greatly increased viscosity of the reaction mass.

In the present study the size of the vagina was found to increase in various degrees after natural and certain artificial inseminations. The increase of size at the maximum reaction was greater for natural insemination and testes brei injections than for the other materials. The reaction mass is present in the vagina of natural insemination, testes brei injection, testes plus male accessory gland brei injection. The vaginae which increased in size significantly also showed an increase of the volume of epithelial cells, but degree of increase is not the same in all cases. The vaginae of natural insemination and testes brei undergo the maximum increase of cell volume, a lesser degree was found in the testes plus accessory gland brei injections and a still lesser degree in the saline injections.

At first sight it may seem surprising that the vaginal epithelial cells increase in volume, especially since distention of the vagina makes the vaginal wall become thinner. But it should be remembered that the vaginal wall is only an invaginated part of the general body integument, and that it is well known that the epidermis of arthropods increases greatly (common range 2–5 times in thickness) during the period when a new cuticle is being deposited (Wigglesworth, 1939). The reaction of the vaginal wall, then, seems quite in keeping with reactions to be expected of arthropod epidermal cells.

The vaginae with a reaction mass have their epithelial nuclei somewhat dense in appearance. In other cases, even though the vagina may increase significantly in size, as in saline injections, the reaction mass is absent, the nuclei generally show diffusely oriented chromatin threads as those of the virgin female, and the cytoplasm is more lightly stained.

In mated females the vagina increases in size after mating (Patterson) and therefore the reaction mass must contain both semen and material added to it. Presumably the material added to the semen is secreted by the vaginal epithelium but origin from associated organs is not excluded. In addition the cells of the vaginal epithelium increase in size and stainability; therefore they too have material added to them. In response to the stimulus from the semen, these cells must absorb material from the blood (and perhaps alter this metabolicly). If these cells secrete the material into the lumen of the vagina, then they must absorb from the blood enough to account for the increase in both the reaction mass and in the cells. Obviously there is a strong reaction by the cells to the stimulus afforded by the presence of semen (or some component in the semen).

The reaction brought about by mating and by testes brei or testes plus male accessory gland brei injection reveals all the features just mentioned. However, in the specimens injected with saline, although the size of the vagina and the volume of epithelial cells increase, the nuclei are loose and the cytoplasm is lightly stained and the reaction mass is never present. From the evidence that the cytoplasm is lightly stained it is assumed that increase of cell volume is simply the result of the uptake of water from the injected saline on account of the lower osmotic pressure of the saline used as compared with the protoplasm of the epithelium. In order to make a more accurate comparison of the staining susceptibility of cytoplasm of vaginae after various treatments, sections of vaginae from a mated female, a female of saline injection and a virgin female were arranged on the same slide so as to obtain a uniform staining process. The results show that the cytoplasm of vaginal epithelium from the mated female is more heavily stained by hematoxylin and eosin than that from the female of saline injection, while that of the virgin female has a staining susceptibility intermediate between those two. Obviously, the cytoplasm of vaginae injected with saline is diluted and the dilution is almost certainly due to the uptake of water from the saline solution injected. The contraction of vaginal epithelial cells by injection of 2 per cent NaCl contributes further support to this assumption, because the osmotic pressure of 2 per cent NaCl is generally higher than that of the protoplasm of insects.

Since the injection of male accessory glands did not induce any insemination reaction in the present data, it is assumed that the secretion of the male accessory gland is not the substance to initiate the reaction. Quantitative effects are not yet precluded but it seems most reasonable to conclude tentatively that the reaction is due to some semen component derived from the testes. As Patterson presented some evidence that the spermatozoa are not the active agent to induce reaction, the inducing substance may be a factor in the testes besides spermatozoa. The active component is not yet known, and we have only two clues about it: (1) it is probably from the testes, and (2) its action suggests protein.

Summarizing the material presented in this discussion: The insemination reaction can be broken down into five component parts: (1) an increase in size of vagina, (2) an increase in volume of epithelial cells, (3) the presence of a reaction mass, (4) a dense appearance of nuclei, and (5) heavily stained cytoplasm. To a certain extent these are experimentally separable. All are produced by mating and by the injection of brei containing testes. (1) plus (2) can be produced by hypotonic saline solution; (4) plus (5) can be produced by hypertonic saline solution; (1) alone is produced by osmotically inactive solutions. It would seem likely that (3) alone could not be produced, *i.e.*, that the reaction mass necessitates the other component steps, but this cannot be proved until the active agent is available for artificial insemination.

SUMMARY

1. Macroscopic features and microscopic structure of vaginae of *Drosophila gibberosa* Patterson and Mainland are described in virgin females, mated females, females with testes injection, male accessory gland injection, testes plus male accessory gland injection, whole male brei injection, 2 per cent gelatin injection, saline injection and 2 per cent NaCl injection.

2. The wall of the vagina consists of intima or cuticle, epithelium and muscle layers.

3. The insemination reaction occurs in vaginae of mated females, females with testes injections, and testes plus male accessory glands injections. The reaction consists of five characteristic features: (a) increase of size of vagina, (b) increase of volume of epithelial cells, (c) presence of reaction mass, (d) dense appearance of nuclei, and (e) more heavily stained cytoplasm. Some but not all of these features have been produced separately by artificial insemination with experimental solutions.

4. The increase in volume of the vaginal epithelial cells is compared with the known increase in volume of epidermal cells during deposition of a new cuticle.

5. Cell volumes can be altered by the injection of hypertonic and hypotonic saline solutions as shown by both volume and intensity of staining.

6. The reaction of vaginae of mated females reaches its maximal distention two and a half to four hours after mating. Then it decreases in size gradually and returns to its normal size five or six hours after mating.

7. The reaction mass within the vagina is composed of spermatozoa and substances of acidophilic staining property. The increase and decrease of the size of

the reaction mass during the course of the reaction keeps well in pace with the size of the vagina.

8. The insemination reaction of vagina as demonstrated by Patterson (1947) seems not induced by spermatozoa, and the present data show that it is not induced by male accessory glands either. Since the semen of Drosophila consists of secretion of accessory glands, spermatozoa and some substances from testes besides spermatozoa, it is suggested that the agent which induced the insemination reaction is most likely some factor from the testes other than the spermatozoa.

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NOTE ON THE GROWTH OF ARMS IN THE PLUTEUS OF ARBACIA PUNCTULATA

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I

Allometric studies have dealt extensively with invertebrates whereas descriptions of growth in which time is explicit rest largely on measurements of prenatal and postnatal vertebrates. In logarithmic form the allometric equation $y = bx^a$ predicts a straight line (Huxley, 1932). Glaser's (1938) linear $\log w = k \log (2T + 1) + C$ as Kavanagh and Richards (1942) have shown is essentially a variant of the allometric equation in which weight, w, can be equated with y; the time factor with x, while $k + C \log 2 = \log b$. Both types of analysis have been subjected to rigorous criticism. Thus Kavanagh and Richards (p. 168) point to "varying degrees of concavity or convexity, including occasional approximations to linearity." Among the case of "upward concavity" they cite Figure 11 (Glaser. l.c., p. 41) which happens to illustrate the nutritive disturbances that characterize the weaning period in mice. However, there are also "a few examples of linearity." Those specifically cited by Kavanagh and Richards refer to the growth of fresh weight in the chick; to the growth of embryonic chick brain; and to the accumulation of such chemical entities as arginine, cystine, and purine. Many of the data dealt with by Glaser are subject to considerable experimental errors and no doubt their treatment could be improved, yet even so it is permissible to ask whether cases where a single stanza of growth is admittedly linear are not as significant as those to which objection is made. This query is especially pertinent since the two stanza treatments result in correct orders of magnitude for the allantoic removal of Ca from the shell (Glaser, p. 27), and also enable us to account within 247 mgs. for the total protein metabolism of the developing egg (Glaser, p. 31).

II

We now turn to E. B. Harvey's (1949) measurements on the growth of the long anal arm of the *Arbacia pluteus*. Here we have an adequately timed invertebrate material. Like all the others, this arm is a definite organic entity whose lengths we may consider proportional to a series of weights (themselves controlled by increases in the lengths of skeletal rods) somewhat diversified in structural detail but probably uniform in chemical composition. If valid, increasing arm lengths would involve proportionately increasing amounts of essentially the same organic categories. How closely then does the growth of this structure follow the linear law?

Harvey's cultures were fed on diatoms. However, even though derived from the same batch of eggs, individual plutei and their arm lengths (measured in microns) proved quite variable. The data therefore are averages based on selection of the better developed specimens. Since nutrition markedly affects arm lengths, especially in plutei older than 4 days (Harvey, l.c., table, p. 294) we can assume that selection influences the uniformity of the measured material by segregating comparable samples of the best fed individuals.

Developmental time, recorded in days, weeks, or months, and now reduced to days, does not include pre-pluteal time. This introduces an error in timing, but the error is reasonably constant and if corrected, would only improve the distribution of the points. These are plotted in Figure 1 according to the modified allometric equation.

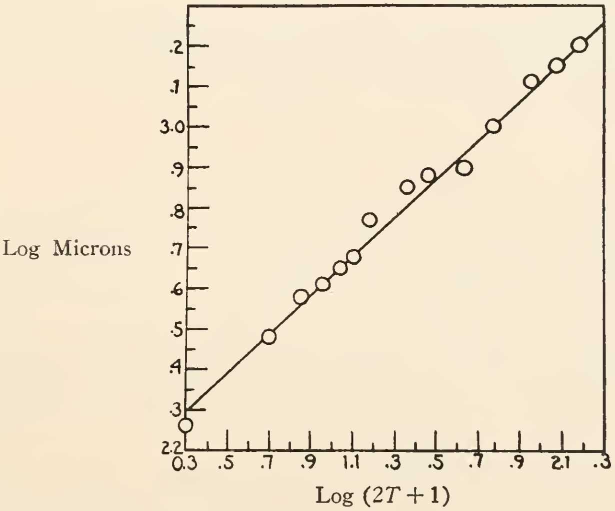

FIGURE 1. Modified allometric plot of growth in length of anal arms in the pluteus of *Arbacia punctulata.* Original data of Ethel Browne Harvey.

As the figure shows, arm lengths for the first and twenty-first days are less than the equation demands while those of the 7th, 11th and 14th days are greater. Since the 14th and 21st days fluctuate about the line, we can attribute these two deviations to chance. Whether the 7th and 11th days represent systematic deviations may be an open question. Certainly the remaining points closely approximate the predicted straight line. It would be curious if this approximation were attributable to the accidents of a selection involving judgement and made for other purposes. Thus in spite of criticisms some of which may be valid, the modified allometric equation seems to apply to a wide range of growth measurements.

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REGENERATION IN AN EARTHWORM, EISENIA FOETIDA (SAVIGNY) 1826. II. POSTERIOR REGENERATION

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This contribution is the second in a series presenting the results of an attempt to obtain, for one particular species of earthworm, complete characterization of regenerative capacity with reference to exact levels. In this part, posterior regeneration, and by anterior substrates only, is considered.

Summary of Previous Work

The information hitherto available as to posterior regeneration by anterior substrates of *E. foetida,* is summarized in Table II. Morgan once referred to one posterior regenerate, a monstrosity, as "a double tail." Other posterior regenerates of various workers from Morgan (1895) to Moment (1946) were not definitely characterized as to morphological nature. Even after rediscovery of anterior heteromorphosis in 1898–99 (see part I) no possibility of posterior heteromorphosis seems to have been anticipated. Very little evidence is available that might indicate morphological nature of many regenerates and the most useful clue, number of segments, may be of little value in certain circumstances.

The uncertainty as to nature of regenerates, paucity of data at most of those levels where operations were made, and lack of data for so many other levels, indicated need for a systematic investigation of regeneration at each level.

Material, Methods and Nomenclature

In addition to the information in corresponding sections of part I, only a few comments are necessary.

Especial effort was made to avoid use of animals that had undergone amputation followed by enteroparietal healing without regeneration (see Gates, 1949b).

Previous work on other species had shown that accumulation of ingested material, such as earth or manure, which could not be passed out by the operated animal, frequently resulted in rupture. Regenerates of survivors were always deformed. To obviate those abnormalities and the higher mortality, as well as effects of pressure of accumulations on the regenerating region of worms which did not rupture, operated animals were at first kept in filter paper or paper towelling. Fibres of such materials were, however, ingested by some individuals in amounts sufficient to result in rupture. This always proved fatal, in *E. foetida,* within 48 hours. A fine grade of cheesecloth was then used. Edges of the cloth had to be hemmed so that free ends of threads were not exposed, as experience with that material, as well as with coarse towelling, showed that worms were able to pull

TABLE II

Posterior regeneration in Eisenia foetida

Level	Number of regenerates				Number of segments in regenerates	Comments on		Time days*	Author	Date	Page
	H	U	T	O		substrate	regenerate				
3/4				5				−6	Michel	1898	261
4/5				5				−6	Michel	1898	261
5/6				5				−6	Michel	1898	261
6/7	(1?)				+			?	Korschelt	1898	79
7/8				5				−6	Michel	1898	261
	(1?)				+			?	Korschelt	1898	79
8/9				5				−6	Michel	1898	261
	(1?)				+			?	Korschelt	1898	79
9/10	(1?)				(7)	6s+"3 angeschnittenen segmenten" See note (a)		?	Korschelt	1898	80
10/11		1			0		Bud	20	Korschelt	1897	100
11/12				5				−6	Michel	1898	261
12/13				5				30	Morgan	1895	458
				5				−6	Michel	1898	261
13/14											
12/13+?	(1?)				4	12+"einigen verletzten segmenten"		?	Korschelt	1897	100
14/15				5				40	Morgan	1895	458
15/16				15				60	Morgan	1897	571
16/17				4				?	Morgan	1895	458
				1				7	Hescheler	1896	234
17/18				1				7	Hescheler	1896	234
				5				−6	Michel	1898	261
18/19				1				7	Hescheler	1896	234
19/20				3				40	Morgan	1895	458
				1				7	Hescheler	1896	234
		1					"Knob"	60	Morgan	1906	469
20/21				1				7	Hescheler	1896	234
			3	22	28? ? ?		See note (b) "long posterior ends"	110	Morgan	1897	571
				27				49	Liebmann	1943	600–1
21/22			1		15			?	Korschelt	1898	80
			1		22 "abn"			60	Morgan	1906	465
22/23				3			?	120	Morgan	1895	458

TABLE II—*Continued*

Level	Number of regenerates				Number of segments in regenerates	Comments on		Time days*	Author	Date	Page
	H	U	T	O		substrate	regenerate				
23/24				1				60	Morgan	1895	458
			2		"17–20 abn," 18			60	Morgan	1906	465–9
24/25			1	5			"a half inch"	90	Morgan	1895	458
		4	1	15		See notes (c) and (d).	"with double tail"	110	Morgan	1897	571
		1	2		3+, 12+, ca. 40			60	Morgan	1906	465
				1				10	Korschelt	1896	58
25/26			1		50			60	Morgan	1906	465
				?		Mature		30	Liebmann	1942	378
			?			Immature (age?) regenerated completely		?	Liebmann	1942	378
26/27				6				?	Morgan	1895	458
			2		23, ca. 30			60	Morgan	1906	465
27/28				1				30	Morgan	1895	458
28/29											
29/30											
30/31		maj.	min.		20–30 mm.		See note (e)	30	Liebmann	1943	602
31/32			1		25			60	Morgan	1906	463
32/33			1	2	72			90	Morgan	1906	463–73
33/34			3		13 (abn) 45, 54			60–90	Morgan	1906	463–73
34/35			3		30+52, 53	See note (f)		60–90	Morgan	1906	463–73
35/36			1		45			60	Morgan	1906	463
36/37			1		60			90	Morgan	1906	473
37/38			1		37			60	Morgan	1906	463
38/39			1		52+			30+	Morgan	1906	464
40/41			7		47–56	See note (g)	Mean = 52 S	30+?	Moment	1946	496
50/51			180		22–57		Mean = 39.5 S	30+?	Moment	1946	496
60/61			19		19–38		Mean = 29.6 S	30+?	Moment	1946	496
70/71			31		6–31		Mean = 21.3 S	30+?	Moment	1946	496
80/81			118		2–22		Mean = 9.8 S	30+?	Moment	1946	496
−L 20S			5		6–7–9–15–19	See note (i)		26	Michel	1898	260
					6–7–13–17–20		Same regenerates two weeks later	40	Michel	1898	260
−L 10S			4		5–5–6–7			26	Michel	1898	260
−L 12–10S			?		"a few segments"			?	Morgan	1906	471
−L 5S			5		3–4–4–5–6			26	Michel	1898	260

off and swallow lint in sufficient quantity to result in rupture of posterior end of substrate or the regenerate.

Use of starved or starving animals, in addition to reducing mortality, also provided greater uniformity in experimental conditions, for, if food was available, anterior substrates, having mouths, often did eat, while posterior substrates from the very same animals, having no mouths, could not eat.

Anterior substrate refers to any "cephalized" portion of the body, regardless of size, comprising the buccal region and extending back to a single posterior transection.

Summary of Results

Healing in some individuals was enteroparietal rather than cicatricial. Such specimens did not regenerate in the two or three months before experiments were

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Notes to Table II

H—Cephalic
U—Uncertain
T—Caudal
O—None
S—Segments

* Time may indicate day of death of last specimen, of last observations, or of termination of experiment. In case of Morgan's experiments time is approximate—because of infrequency and irregularity of examination of operated animals, and method of presentation of data.

(a) The cut was diagonal and from the data provided in the text as quoted above, level of regeneration, in accordance with previous practice (see Part I, Table I, note c), should be taken as 9/10. However, Figure 8 shows a substrate of at least 7, possibly 8, complete segments and major portions of two others (missing portions apparently not regenerated). Level of regeneration, according to the figure, should, perhaps, be taken as 10/11.

The figure does not have, in the seven-segment regenerate, the terminal notch that indicates, in certain other regenerates on the same page, presence of an anus.

In *Regeneration and Transplantation* (1907, p. 80, fig. 50B) the same substrate appears to have been reproduced but the regenerate, again without a terminal notch, is marked off into eight divisions, the last of which could represent a segment or a prostomium. Alongside (in Fig. 50A) is shown a substrate of 11 presumably complete segments with a regenerate of nine segments of which the terminal is notched (1927, p. 332, Fig. 188). If 50A is a representation of an actual specimen, caudal regeneration anterior to 20/21 is indicated.

(b) Figure C (Morgan, 1901, p. 8), shows a 20-segment substrate with a regenerate of 28 segments. Although 20/21 is the anteriormost level of regeneration definitely recorded by Morgan, two statements seem to indicate regeneration further forward.—"Anterior ends containing from thirteen (?) to thirty segments sometimes regenerate posteriorly" (summary, 1897, p. 584), and "as I have shown elsewhere, the power of regenerating a posterior end ceases rather suddenly about the level of the 15th segment" (1906, p. 463). In both, reference presumably was to a doubtful case of regeneration (posterior) at 13/14 by a two-surfaced fragment and not an anterior substrate. Note also "no cases of survival of as few segments as fifteen were ever found" (1895, p. 458).

(c) Number of segments in substrate, according to text, 25 or 24, according to table (I, p. 571), 24. The latter is taken as correct as Figure 1 shows 24 segments in the substrate.

(d) Figure 1 (Morgan, 1897) shows a regenerate with numerous metameric abnormalities, bifurcated distally, one bifurcation represented as having an anus. This is the posterior regenerate that was morphologically characterized.

(e) "The majority die after regenerating short pieces" at least some of which may well have been cephalic. "Those which survive do reform full size individuals."

(f) At EL 34/35 eight regenerates: abn., 19 (abn), 31, 37, 36–40 (abn), 40 (abn), 55 (abn), 61. Time, *ca.* 60 days. Actual level of regeneration could have been from 31/32–38/39.

(g) Morgan (1906) experimented in two regions behind 40/41; at the "middle" of the body (EL 50/51) and after excision of L25–20S (EL 75/76–80/81). Actual levels, when deterterminated (but see note (h), Table II). In other specimens a small bud was formed. At certain anterior levels, the bud shortly ceased to grow. At more posterior levels, further growth often enabled recognition of a growth zone producing segments by the rapid ventral or terminal methods (see Gates, 1948). In the former case, the ventral method eventually gave way to the terminal and finally the growth zone was reduced to a very small anal region, as in normal worms. Thereafter new segments were formed by the slow terminal method of normal, late-juvenile or post-juvenile growth. Regardless of length of time animals were kept after operation the regenerate always remained recognizable as such and clearly distinguishable from substrate.

Posterior regeneration, at levels behind 40/41, was obtained throughout the entire year.

A. After a single cut

Anterior substrates of 15 or fewer segments did not regenerate and in many cases probably did not live long enough to do so. At levels 16/17–19/20 small buds were formed but soon ceased to grow. These buds were still unpigmented, metamerically undifferentiated and without terminal sculpturing 80–90 days after operation when experiments had to be terminated. The external appearance of such regenerates was more like that of a cephalic than a caudal bud.

Regenerates (metamerically organized growths) were obtained at each intersegmental level from 20/21 posteriorly. In the Cambridge experiments percentage of regeneration gradually increased from 20/21, with 100% regeneration on substrates having transections at levels 32/33–39/40. With but two exceptions, all regenerates were caudal.

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mined, were at 41/42–64/65 and 64/65–88/89. "In order to compare the rate of regeneration at the beginning and toward the end of its period of growth some of the same lot of worms that gave the records of Table IV were kept alive for another month (Jan. 6 to April 6) in order to see how much further the regeneration of new segments would continue. The results are given in the following Table IX" (1906, p. 473). But the worms of Table IX were "Cut behind girdle" as proved by number of segments, "at middle" and near posterior end, while none of the worms in Table IV were cut near the posterior end nor behind the girdle. Accordingly the comparable results were those of the two sets of regenerates at EL 50/51. Omitting from consideration those substrates in which segment number was not determined, the results appear to be as follows.—Two months; four substrates of 41, 47, 55, and 56 segments with regenerates respectively of 58, 44, 43, and 39 segments, average number of segments 46. Three months; seven substrates of 41, 48, 51, 51, 52, 53, and 59 segments with regenerates respectively of 53, 52, 46, 40, 36, 42, and 32 segments, average number of segments 43.

(h) Morgan's Table I (1897, p. 571) seems to indicate that regeneration at levels 20/21 and 24/25 began only in the second month after operation. (Also *see* second paragraph on p. 582.) Korschelt, however, obtained an unsegmented bud at 10/11 in 20 days and mentioned similar "Knospen" on other substrates presumably of the same series. Hescheler (1896, p. 242) found that the anterior half of an autotomized specimen of *Allolobophora chlorotica* (Savigny) 1826, regenerated only after nine months (autotomy in November, regeneration in August).

Liebmann (1942) apparently found no effect of season on posterior regeneration in *E. foetida* though other species failed to regenerate in winter months. In 31 October operations at 12/13–26/27 (Morgan, 1895, p. 458) no regeneration was obtained, while in 8 January operations at 19/20–27/28 one regenerate was secured. No data available as to season of Morgan's 1897 operations and Korschelt's 1898 operations. Korschelt's bud at 10/11 was obtained in August.

(i)—L20S Level of cut when last twenty segments were excised.

In the first exceptional case the small regenerate, produced in 65 days at 32/33, had the external characteristics of an inverted buccal segment, the prostomium ventral rather than dorsal. The second exceptional regenerate, produced at 34/35 in 65 days, was differentiated into six segments of which the proximal five were normally setigerous. The terminal segment had all the external characteristics of a normal buccal segment with normal prostomium.

At each level from 92/93 to 124/125 one, two, three, or ten segments were removed. Every specimen (100 +) regenerated. Regenerates produced after removal of ten segments were hypomeric, as at levels anterior to 92/93. After removal of one to three segments, regenerates were usually equimeric or hypermeric. In one specimen, number of segments in regenerate and substrate totalled 126, one more than the maximum hitherto recorded for the species.

Attempts at removal of a portion only of the so-called anal segment also resulted in hypermeric regeneration.

B. *After a previous regeneration*

Attempts to test for effects of previous regeneration were made in several series. In E13 and E14, the first two and three segments were removed and the substrates were allowed to regenerate for 14 days, at which time the worms were depositing faecal pellets of paper indicating that the digestive system was functional. Two days later posterior portions were removed at 8/9 or 15/16 (all anterior regenerates equimeric). All substrates of eight segments died without regeneration. Three fifteen-segment substrates survived until preservation 65–95 days later. Each had a regenerate at 15/16. One was abnormal. The second, though two mm., long, was metamerically undifferentiated and with the terminal portion sculptured into the shape of an inverted buccal segment as in the exceptional regenerate at 32/33. The other regenerate was marked off into four segments but setae were unrecognizable externally and the terminal portion was unsculptured (no indication of prostomial demarcation, buccal or anal invagination).

In E43 the anteriormost eight segments were removed. Six worms survived this operation. Forty-five days after the first operation, posterior portions were removed at 35/36. Three survivors of the second operation regenerated in the time available. No differences were noted between those regenerates and others produced at 35/36 by substrates that had not previously regenerated.

C. *After starvation*

Worms that had been starved seemed to regenerate as well as those which had not been starved prior to operation. As a result of several months starvation the chloragogue layer on the gut of substrates was drastically reduced. The masses of coelomic corpuscles which had been readily visible in young tail regenerates of animals operated upon shortly after collection, were not noted in those regenerating after weeks of starvation.

Discussion

A zero level posteriorly for tail regeneration apparently has been indicated by Liebmann (slightly to left of c, 1943, p. 601) and by Moment (at 90/91, by extrapolation in curve of Fig. 2, 1946, p. 406). New results presented above show there is no zero level posteriorly.

Liebmann and Moment apparently also found that production of new segments ceased at or shortly after the first month. The author's results agree with those of Michel (1898) and some of Morgan (1895–1906), in showing that production of segments may be continued longer than a month. After reduction of the regenerative growth zone to the small anal region characteristic of the normal adult, further segment formation apparently is continued by a slow terminal method.

Establishment in tail regenerates, at the same level and in the same experimental conditions, of different types of growth zone (ventral, large or small terminal, see Gates, 1948), with different capacities for segment formation, suggests that the variation may be due to the condition of the operated animals (note also considerable variation in number of segments regenerated at each of Moment's levels, 1946, p. 496).

In favorable conditions, and at levels from 40/41 backwards, season apparently has no influence on ability to regenerate posteriorly (by anterior substrates) in *E. foetida,* although seasonal inability to regenerate posteriorly has been reported from other Lumbricid species by Liebmann (1942, for *Lumbricus terrestris*)[1] and by Abeloos and Avel (1928, for *Allolobophora longa* and *A. terrestris*). Effect of season on posterior regeneration at levels in front of 40/41, in *E. foetida* as well as other Lumbricids, remains to be determined (see note h, Table II).

The hypomery of tail regenerates at anterior levels and after removal of ten or more segments posteriorly, in the author's experiments, is in accord with D'Arcy Thompson's rule "that regeneration tends to fall somewhat short of a *complete* restoration of the lost part; a certain percentage only of the lost tissues is restored" (1942, p. 274). Equimery and hypermery at levels behind 92/93, when three or fewer segments were removed, provide, in earthworms, a second exception to the rule.[2] Michel (1898) had a single case of hypermery after removal of five segments (see Table II), but no information is available to indicate that the specimen was normal and complete at time of amputation. Moment (1946) obtained some hypermery in tail regenerates at 50/51 and 80/81, apparently mostly from specimens with only 80–89 segments, possibly also in some with 90–99 segments. As number of segments in juveniles 7.5 + mm. long may be 85–115 (Gates, 1948), and usually is somewhat over one hundred by six months (Liebmann, 1943, p. 600), Moment's worms with the smaller numbers of segments, 80–89 or even 90–99, may have been amputees, possibly with enteroparietal healing and no regeneration (see Gates, 1949b).

Posterior heteromorphosis, i.e., regeneration of a head at the posterior surface of an anterior substrate, hitherto has been unrecognized in the Lumbricidae. Such heteromorphosis has, however, already been recorded from species of two other families of earthworms, in the Glossoscolecid *Criodrilus lacuum* by Janda (1926) and in the Megascolecid *Perionyx excavatus* by Gates (1927). In that respect, at least, pattern of regenerative capacity now appears to be similar in the three families.

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[1] There are no valid records of posterior regeneration by this species in any season!

[2] The first exception; equimery of anterior regenerates in the anteriormost portion of the axis (see part I).

Posterior heteromorphosis having been recognized in *E. foetida,* it is now possible to suggest cases in which similar results may have been obtained by previous investigators, though unrecognized. Among such cases may be mentioned: the "majority" of Liebmann's animals operated at 30/31 which died "after regenerating short pieces," and Korschelt's regenerates at 12/13 (+ ?), 9/10 (or 10/11), 8/9, 7/8 and 6/7 (see Table II). In fact, the only evidence available to indicate that posterior regenerates in *E. foetida,* at levels anterior to 20/21, are other than cephalic, is Korschelt's figure (1907, Fig. 50A, see note (a), Table II) of a specimen about which nothing else is known. In spite, then, of the failure of the present and some other authors to obtain any regeneration anterior to 20/21, there appears to be a possibility of heteromorphosis as far forwards as 6/7. This is supported, to some extent, by the heteromorphic heads regenerated by reversed grafts[3] in three different Lumbricid species: *E. foetida* (Hazen, 1899), *Allolobophora longa* (Ruttloff, 1908, and Leypoldt, 1910), and *A. caliginosa* (Crowell, 1937).

Cases of heteromorphosis from reversed grafts have all been at rather anterior levels, 7/8 (Hazen and Ruttloff), region of 8/9–7/8 (Leypoldt), 12/13 (Crowell). Levels of heteromorphosis, involving a single transection only, were somewhat more posterior. The level for Janda's case, in *C. lacuum,* was 22/23. The level for *P. excavatus* was not published, but almost certainly was in front of 20/21 and probably behind 12/13. Formation of heteromorphic structures in *E. foetida,* as far back as 34/35, accordingly had not been anticipated, though expected in a more anterior region where no regeneration (except uncounted, inhibited buds) was obtained by the author.

Regeneration of heteromorphic heads at levels 6/7, 9/10, 32/33, 34/35, etc., is believed to indicate similar regenerative ability at all of the intermediate levels.

Number of segments in heteromorphic heads on reversed grafts usually has been small for the level: 2 segments at 7/8 (Hazen), 3 in region of 8/9–7/8 (Leypoldt), 3, 4, and 5 at 7/8 (Ruttloff), unrecorded for Crowell's regenerates. However, Janda's regenerate, following simple transection only, at 22/23, had 16 segments, and Korschelt's at 9/10 had seven segments. Homomorphic head regenerates may have 16 segments in *C. lacuum* and nine segments (the maximum hitherto recorded for the Lumbricidae) in *E. foetida* (see part I). It would not be surprising if further work demonstrates an ability to produce at any particular level, in the proper conditions, as many segments in heteromorphic as in homomorphic head regenerates.

Regeneration of a heteromorphic head at 34/35 where a homomorphic tail may also be regenerated, indicates for *E. foetida* a region of bipotential regenerative capacity for posterior regeneration, such as has already been demonstrated in part I for anterior regeneration. For the present the posterior limit of the region is placed at 34/35 by the regenerate just mentioned above. The anterior limit is placed at 20/21 by the foremost recorded level of homomorphic regeneration. The region of bipotential capacity for anterior regeneration, as delimited in part I, is between 35/36 and 18/19. The two regions are so nearly coextensive as to suggest possibility of identity.

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[3] At levels anterior to 20/21, regenerates from reversed grafts have usually been heads which must be considered heteromorphic with reference to original orientation of cut surfaces. The single exception was a regenerate that "erwies sich bei mikroskopischer Untersuchung als Hinterregenerat" (Ruttloff, 1908, p. 471), at 7/8 in *L. terrestris.* This case (Ruttloff's No. 60), is also of especial interest because of lack of valid records of tail regeneration by *L. terrestris* from ungrafted substrates.

Regeneration of heteromorphic heads anterior to 20/21, and regeneration of such heads behind 20/21, is not in accord with the polarization of the head aggregate and the specificity of the eleocytic aggregates postulated by Liebmann (1943).

SUMMARY

Anterior substrates of *E. foetida,* cut exactly at intersegmental furrows, regenerated homomorphic tails at levels from 20/21 back, with no zero level posteriorly. Regenerates were hypomeric, except posteriorly and after removal of three or fewer segments, and then were equimeric or hypermeric. Heteromorphic heads, previously unrecognized in the Lumbricidae, were obtained at 34/35, 32/33, 15/16 (but only after a previous anterior regeneration). Head-like buds were formed at 16/17–19/20.

Starvation and season did not inhibit homomorphic regeneration at levels behind 40/41.

Posterior regenerative capacity in a region from 34/35 to 20/21 is characterized as bipotential, as a posterior regenerate in that region may be a homomorphic tail or a heteromorphic head.

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THE LEUCOCYTES OF ARBACIA PUNCTULATA

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Geddes (1880), whose studies supersede earlier investigations, distinguished two main types of leucocytes[1] in sea-urchins: colorless, hyaline or granular elements and colored ones with the pigment attached to cytoplasmic inclusions. He found the first to be concerned with clotting and with phagocytosis of foreign matter, observations repeatedly confirmed later; the second were surmised to serve respiration. Cuenot (1891) observed that certain granulocytes of sea-urchins liberate their albuminoid or fatty inclusions throughout the organism and claimed that these granulocytes develop from the hyaline elements (or within them), as the latter take up nutrient matter from the digestive system and transform it into the cytoplasmic granules. His later studies (1897) brought him to entirely different conclusions, viz.: that the cytoplasmic inclusions represent end products of metabolism, absorbed and eliminated by these cells, a view also held by Delage and Herouard (1913). Kindred (1924, 1926), however, supports the earlier views of Cuenot. The close connection of the granular leucocytes with the digestive tract and their penetration into its lumen through the intestinal epithelium is interpreted as a secretory function by Frenzel (1892).

Saint-Hilaire (1897) drew a sharp line between the agranular phagocytes, which he observed to ingest foreign matter and dead cells, and the granulocytes. He confirmed earlier observations regarding the abundance of the latter in and around the intestinal wall but found no indication that they are concerned with nutritive processes. A search for granulocytes in the gonads revealed no great numbers in them and no clue as to their function, leading him to the then prevailing notion that they help eliminate waste products.

The red pigment of the granulocytes, named echinochrome by MacMunn (1885), is claimed by him to be an oxygen carrier, a view shared by Griffiths (1892). Cannan (1927) found it to be an activator rather than a carrier of oxygen. Crude extracts of echinochrome are claimed by Friedheim (1932) to increase the respiratory rate in sea-urchin eggs; this, however, is denied by Tyler (1939) for the chemically pure substance. Echinochrome was also found to be present in the eggs and test of *Arbacia punctulata* (McClendon, 1912).

Comparative hematological studies on representatives of tunicates, insects, annelids and the coelenterate *Tubularia* (Liebman, 1946, 1947 *et ante*), have revealed two main types of leucocytes in these organisms: ameboid, agranular *phagocytes,* and non- or poorly ameboid, granular elements having a nutritive function and hence termed by the writer *trephocytes*. Each kind was found to exhibit distinctive morphological and physiological characteristics, having frequently a separate locus of origin. The present investigation disclosed that conditions in Arbacia do not differ fundamentally from those found in other invertebrates studied and thus an extension of the scheme outlined above is permitted.

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[1] This term is applied to all blood cells excluding those containing hemoglobin: the erythrocytes. "Granulocytes" designates elements with endogenous, regularly occurring cytoplasmic inclusions, irrespective of their size.

Material and Methods

Specimens for cytological studies were collected and preserved at the Woods Hole Marine Biological Laboratory during June and July, 1945. Additional material was secured and observations in the living state were made at the end of July, 1948. All were performed on fully grown, apparently healthy, individuals.

The leucocytes of Arbacia undergo profound changes during fixation and in some cases disintegrate beyond recognition. In addition, the pigments which characterize the various kinds of trephocytes dissolve in the fixatives and usually attach themselves to non-colored or differently colored elements, which easily leads to further confusion and misinterpretations. To avoid these pitfalls, observations on living leucocytes were made the basis of the present investigation. The blood (perivisceral fluid) was consequently studied extensively in the living condition shortly after removal, and also in hanging drop cultures kept at room temperature.

Blood-smears prepared in the conventional way proved of little value. The only method giving satisfactory results was a modification of the formalin-vapor technique described earlier (Liebman, 1945a) : the blood is spread in a thick layer on a slide and placed in a moist chamber, a Petri-dish lined with wet filter-paper serving well the purpose. After 30 minutes the cover of the dish is slightly lifted and a wad of cotton soaked with formalin placed near, but not in contact with, the slide. Fifteen or twenty minutes later the slide is removed, air-dried and stained. The leucocytes, which become active in the moist chamber, are fixed by this method in the characteristic forms as they appear in life. Hanging drop cultures were also successfully fixed by this method. Preparations from blood, quickly withdrawn from the body and injected directly into the fixative, proved useful for some purposes since this method fixes certain elements in the same condition as they are found within the organism; it is referred to in the text as "whole-blood" method.

Preparations showing leucocytes fixed *in situ* are preferable where nuclear structures have to be considered. Aside from pigment displacement, mentioned earlier, the leucocytes—especially the granular kind—usually appear here in a contracted, more or less spherical shape, which they seldom assume in life; this, however, proved of considerable advantage when measurements had to be made and these were performed on said material. In fixing tissues or whole organisms, Zenker-formol produced the best results and was used extensively, except when other considerations required different methods.

Wright's blood-stain, strongly diluted in distilled water, and Mallory's connective tissue stain gave the most satisfactory results. Mallory's stain produced particularly sharp pictures of the minute nuclear and cytoplasmic structures, as well as a differential staining of the trephocytes after Zenker-formol fixation.

I am greatly indebted to Dr. Ross F. Nigrelli for his generous help and advice in preparing the photomicrographs.

Observations

A. The trephocytes

Calculations based on a count of 1608 cells from the perivisceral fluid of four individuals showed 50.4 per cent to be trephocytes, the remainder phagocytes. However, the number of trephocytes in the whole organism was probably higher, as they were practically the only leucocytes encountered in the ovaries; reports also agree that they are predominant in the digestive tract.

Observations *in vitro* revealed three kinds of trephocytes, having respectively green, red or colorless cytoplasmic inclusions. For sake of brevity they are referred to here as green, red or colorless trephocytes.

The green trephocytes. These are the least numerous, constituting 10.2 per cent of all trephocytes. They are the smallest in size, with an average diameter of 7 μ and a range of 6–8 μ. The nucleus is small, round and lacks a nucleolus but, in contrast to the two other kinds, it shows minute chromatin granules (Fig. 1, first row). The cytoplasmic inclusions are fine, rather uniform in size and of a lemon-green color in the living cell. They are moderately eosinophilic or, in a small proportion of cases, chromophobic. With Mallory the granules stain reddish-orange, as does the nucleus.

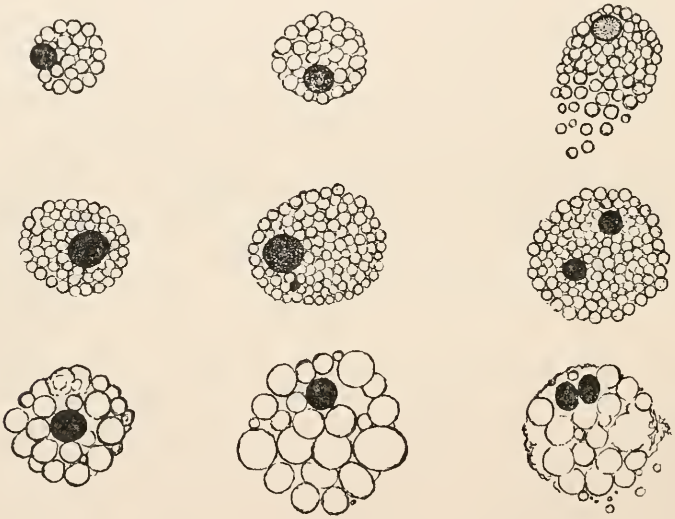


FIGURE 1. Trephocytes from an animal fixed in toto; upper row: green variant; median row: red variety; lower row: colorless variety. Camera lucida drawing; 1650 ×. In each row the element at left represents a young form, that at right an old one.

In the living state, the green trephocytes occasionally contain a number of red cytoplasmic granules like those found in the next variant.

The red trephocytes (Fig. 1, second row) were the most numerous, comprising 48 per cent of the group's total. They average 9.2 μ in diameter with a range of 6.7–12 μ. Their anucleolar nucleus is somewhat larger than that of the green kind, occasionally dumb-bell shaped or fragmented into two portions. When stained, it assumes a flat, structureless aspect. The cytoplasmic granules are uniform in appearance and of the same size as those previously mentioned, but often coarser in the largest elements. In life they are of a brownish-red color due to the presence of echinochrome. They stain light-green with Wright and a brownish-yellow or light blue with Mallory.

The colorless trephocytes comprised 41.8 per cent of the total during the period of investigation. They are the largest kind (Fig. 1, third row), averaging 10.2 μ in diameter with extremes ranging from 6.0–13.4 μ. The nucleus is minute, often smaller than that in the green kind, anucleolar and devoid of structures; in the larger cells it is frequently pycnotic, fragmented or occasionally missing, having apparently dissolved in the cytoplasm. The cytoplasmic inclusions are colorless or faintly greenish in life, irregular in size but predominantly very coarse. They are very strongly basophilic, occasionally slightly metachromatic; Wright's stain makes them appear almost black and completely opaque. With Mallory they stain a light-blue.

Chemical nature of the inclusions. The difficulties encountered in the cytological study of the trephocytes apply also to the cytochemical approach, hence our examinations should be considered of a preliminary character and the information gained rather of a tentative nature. Definite conclusions could only be drawn after a special study of the problem, with new or specifically adapted methods.

Routine techniques did not reveal the presence of osmio- or sudanophilic matter in any of the trephocytes. Orange-yellow coloration after Mallory's staining, assumed by some authors to suggest phospholipins, appeared in the green, red and occasionally also in the colorless inclusions. Glycogen, as indicated by Langhans' iodine-method and Best's staining, and checked with the sputum test, was only very rarely found. The strong basophilia of the colorless inclusions suggests the presence of ribonucleic acid and their positive reaction towards mucicarmin—a mucin. The color of the inclusions of the red trephocytes is generally attributed to echinochrome.

Common characteristics of the trephocytes. The regular presence of endogenous granules is *per se* a common feature of the trephocytes. Another is the characteristic nucleus.

Additional features peculiar to all trephocytes are their function, to be discussed later, and the lack of phagocytic capacity. Observations *in vitro,* both on freshly drawn blood as well as on cultures, in no case revealed ingestion of foreign matter, although in the older cultures extensive phagocytosis was evident in other cells. Similarly, in the fixed material no indication of phagocytosis on the part of the trephocytes was found.

Another common peculiarity of all trephocytes is their way of locomotion. They move by emitting blunt lobopodes into which the granules follow quickly. When moving, the cells usually assume an oblong appearance, frequently with a trail of granules or cytoplasm in their wake (Fig. 2), which gives them a striking resemblance to rat-tailed maggots.

As against these common features, the composition of the granules thus appears to be the main factor distinguishing the three kinds of trephocytes. Even here, however, the limits do not appear to be fast and absolute; this is suggested by some tinctorial as well as cytochemical characteristics common to all granules.

Genetic relationship of the trephocytes. The relatively well differentiated nucleus, the small size and the rather low frequency of senile, disintegrating forms, suggest that the green trephocytes are the youngest of the three variants. Of the green trephocytes, the chromaphobe forms are the smallest, have the largest and best differentiated nuclei and are consequently regarded as the youngest in this group. Also, chromophobia of the inclusions is peculiar to very young cells and has repeatedly been observed by the writer in the earliest developmental stages of granulocytes (trephocytes of annelids, eosinophils of fishes and amphibians). The largest forms amongst the green trephocytes, on the other hand, generally represent the

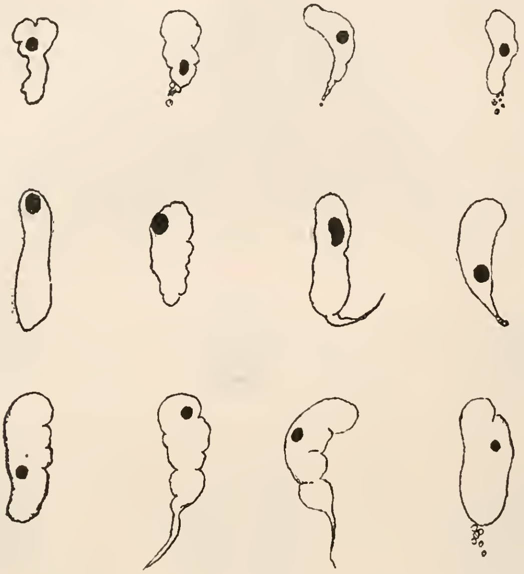


FIGURE 2. Outlines of living trephocytes in motion; from a hanging drop culture; camera lucida; 1000 ×. Upper series: green; middle series: red; lower series: colorless trephocytes.

PLATE I

All figures are unretouched photomicrographs of sections of ovaries fixed in Zenker-formol and stained with Mallory's connective tissue mixture.

FIGURE 1. Cross-section from an ovary, showing the interovular spaces filled with disintegrated trephocytes. The clear areas around the oöcytes are due to shrinkage; 600 ×.

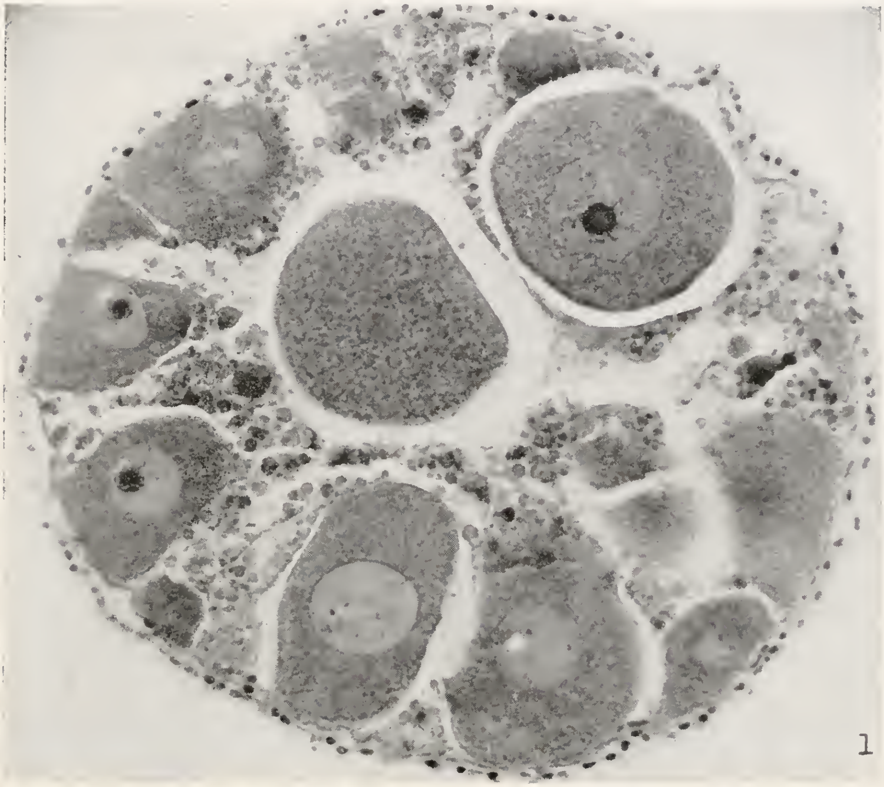
FIGURE 2. A compact mass of partly disintegrated red trephocytes surrounded by oöcytes; the dark spots in the mass represent nuclei; 450 ×.

FIGURE 3. An oöcyte showing uptake of trephocytic granules along its free (upper) side; 650 ×; note lack of cell-wall and continuous stream of granules at the ingesting site. In this, as in the following two figures, the lower edge of the picture corresponds to the site of attachment of the oöcytes to the ovarian wall.

FIGURE 4. A young oöcyte in the process of uptake of larger droplets and a nucleus (along the upper right side); 800 ×.

FIGURE 5. Two oöcytes engorged with trephocytic granules; 800 ×. A close inspection shows small perforations in the ovular walls.

Plate I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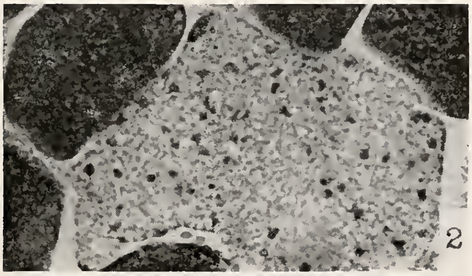
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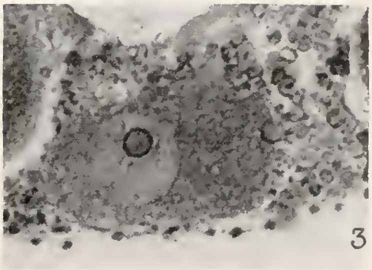
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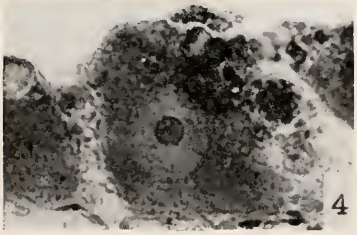
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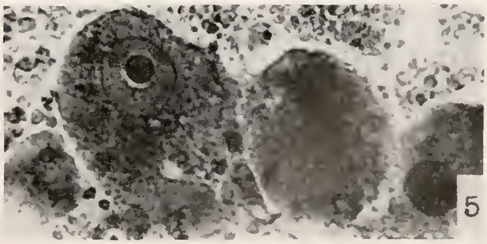
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oldest elements, showing most frequently pycnotic nuclei and signs of cellular disintegration. This scheme, if not too rigidly applied, holds also for the two other variants, the smallest usually representing the youngest, the largest the most mature individuals of each group. Among the three groups, the colorless trephocytes show the most pronounced signs of maturity and disintegration.

Elements which show green and red inclusions are not infrequent, indicating the probable origin of the red variant from the green one. The colorless forms often exhibit a more or less green hue, suggesting also a possible development from the green kind. Tinctorially, on the other hand, the colorless and red granules occasionally show common characteristics. Despite extensive search, however, we have not found in the perivisceral fluid colorless elements with clear-cut transitional or mixed characteristics, as is the case with the trephocytes containing green and red granules. This does not preclude the possibility that the transformation into colorless elements takes place in some organ.

Neither in the blood nor in the tissues studied (gonads and adjoining tissues) were there any indications suggesting development of the trephocytes from phagocytes, nor was there any indication of a common origin.

The function of the trephocytes. Distintegration of all three variants, accompanied by a scattering of the cellular components was observed both *in vitro* as well as in fixed material, in the blood as well as in tissues. This is usually preceded or paralleled by loss of staining capacity of the nucleus or, more rarely, by its becoming pycnotic. Occasionally the cell components simply fuse into one or several droplets; this was observed mostly in the colorless trephocytes. Considering the ubiquity and the high frequency of the processes described, they must be considered as normal phenomena.

The ultimate fate of the trephocytes and their derivatives, as well as the nature of their inclusions, suggest a trophic function. This is particularly evident from their relation to the growing eggs and to the phagocytes, both objects of close study in the present investigation.

The ovaries were found to be densely packed with trephocytes, most of them in a partly or fully disintegrated state. This cellular brei surrounds the oöcytes from all sides except where they border the ovarial wall, as is the case in the youngest elements (Plate I, Fig. 1). It appears to be well attached to the ovules, as witnessed by the difficulty of separating it from the oöcytes in isolates. In some places the trephocyte masses form extensive, uninterrupted islands in the ovary (Plate I, Fig. 2).

Only the red and colorless variants of trephocytes were found in the ovaries. Because of their disintegrated state, it was impossible to submit them to a differential count, the general impression being, however, that their proportion roughly corresponds to that in the blood.

Suitable preparations reveal that the young oöcytes take up granules and droplets of the cellular brei surrounding them. This apparently takes place to a great extent by a process of fusion: the ovular membrane in the areas concerned is very thin, discontinuous or missing and the granules form an uninterrupted stream from the exterior practically up to the nucleus (Plate I, Fig. 3). Occasionally large droplets, some still showing unmistakable marks of nuclei, are found to be taken up in this manner by the oöcytes (Plate I, Fig. 4). On the other hand, very small granules appear to penetrate through the ovular membranes and are frequently found sticking in them (Plate I, Fig. 5). Extremely small perforations are also detectable in the wall of the young oöcytes, as shown in the same figure, but these are not necessarily preformed openings but may represent empty spaces from which granules have been dissolved in the process of fixation, dehydration, etc. The result of all these activities is that the young oöcytes frequently appear gorged with granules (Plate I, Fig. 5), whose physical and tinctorial appearance leaves little doubt regarding their origin from the trephocytes.

The phenomena described indicate that a considerable proportion of the egg's substance consists of material taken up in corpuscular form and derived from the trephocytes. Since a large fraction of this material can be traced to their red variety, the possibility should not be precluded that the echinochrome found in the eggs of Arbacia also has its origin in the trephocytes.

B. *The phagocytes*

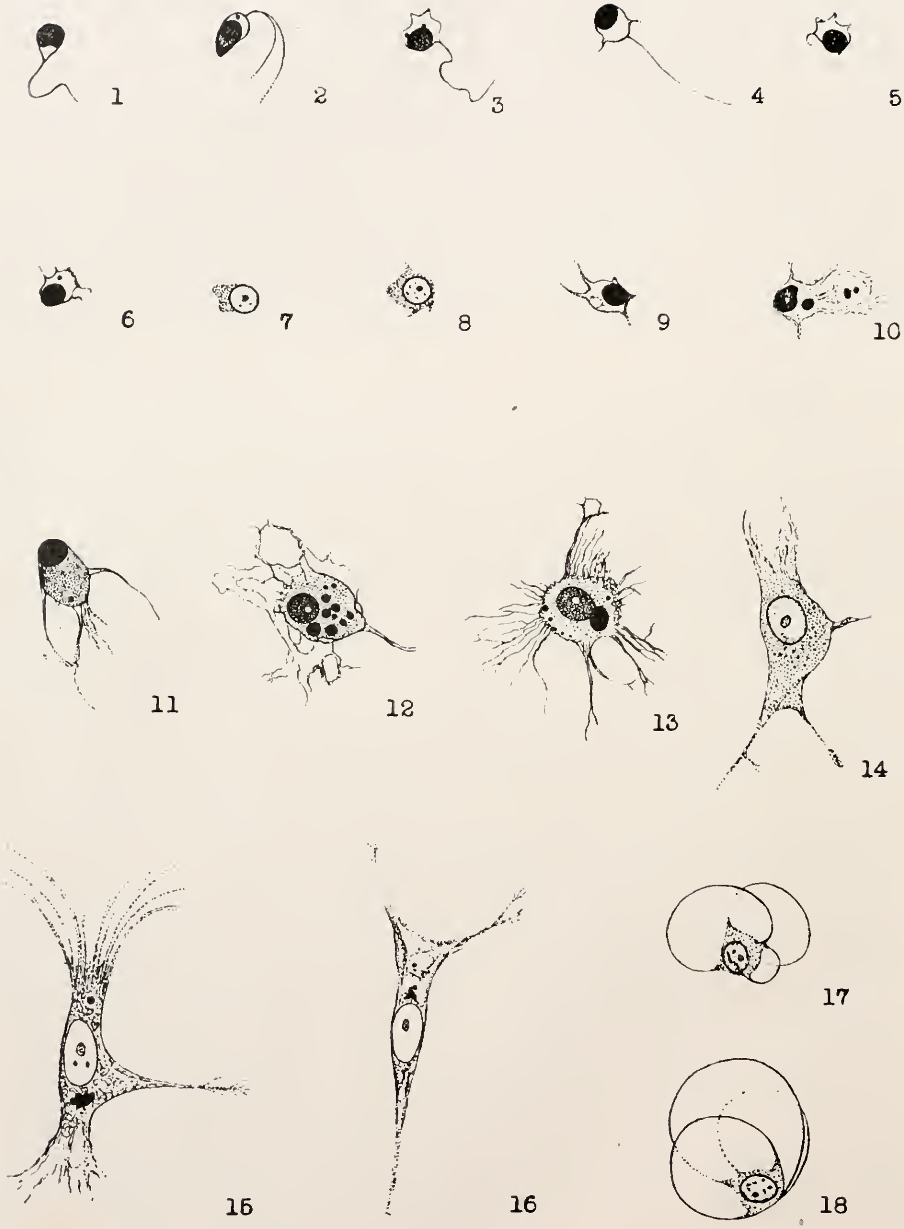
The variety of these elements in the species studied, as well as in the echinoderms in general, has hardly its equal in the animal kingdom. Still, even more clearly than in the case of trephocytes, the present studies reveal that they are merely developmental and functional stages of the same cell, some possibly representing reversible adaptations to the medium in which they are found. They are here classified into four groups, according to their outstanding characteristics: (1) Flagellated forms, (2) Ameboid forms, (3) Fibroblasts and (4) Petaloid forms.

The flagellated phagocytes represent the smallest and apparently youngest stages of this group (Table II, Fig. 1–4). In the living state they appear as spherical elements gyrating and moving briskly among the other blood cells. When fixed, they show one—rarely two— flagella and a spherical, ovoid or piriform cell-body. The nucleus is relatively large and nucleolated, the chromatin fine. (In smears, the nucleolus of these, as well as of other small elements, is rarely visible.) Both *in vitro* as well as in fixed preparations the cytoplasm occasionally shows trephocytic granules, suggesting a certain phagocytic capacity.

Flagellated elements are frequently observed *in vitro* to settle, lose their flagellum and become ameboid. Figures 3, 4 and 5–8 (Plate II) represent such cells prior to and soon after this transition. Except for the absence of the flagellum and the regular presence of small pseudopods, the elements, soon after settling, hardly differ from the flagellated forms. The small size, high nuclear-cytoplasmic ratio, and poor phagocytic capacity, as well as some other characteristics, suggest a similarity of the flagellated and young ameboid forms to the vertebrate lymphocytes.

The ameboid phagocytes. Between the smallest, aflagellar, ameboid forms and the largest elements—the macrophages—innumerable transitions can be observed both *in vitro* and in the fixed preparations (Plate II, Figs. 5–13). They are all characterized by a relatively large nucleus of a round, spheroid, occasionally bean-shaped form, and fine chromatin. A large nucleolus, sometimes two or three, is always present. The hyaline cytoplasm forms long slender pseudopods continuously changing shape and configuration. This ameboid capacity seems to increase with the size of the cells, the latter again being, in general, an expression of their physiological age. Small and medium-sized forms were most commonly encountered in the blood during the present investigation. The whole group cor-

Plate II



Various forms of phagocytes; camera lucida drawings; 1300 ×. Figures 1–6 and 9–16 from a formalin-vapor preparation. Figures 7, 8 from an animal fixed *in toto;* Figures 17, 18 from a whole-blood preparation. The black matter in the cytoplasm represents ingested trephocyte material.

responds to the vertebrate mononuclears; the largest forms show in most respects a similarity to the macrophages of vertebrates and other invertebrates.

The fibroblasts. In hanging drop cultures, elements closely resembling vertebrate fibroblasts (Plate II, Figs. 15, 16) appeared within 24 hours. Transitional forms between them and the macrophages were observed both *in vitro* and in fixed material (Plate II, Fig. 14) and it is highly probable that they develop from macrophages. Without implying that they correspond in all details to their vertebrate counterparts, their general appearance unmistakably justifies their being classified as fibroblasts. The nucleus, except for being elongated, is similar to that of the macrophages; the cytoplasm is fibrillar or reticular, a characteristic appearing also in the macrophages in regions where long pseudopods are formed.

The petaloid phagocytes. Another variety of phagocytes is illustrated in Figures 17, 18 (Plate II). Their ectoplasm forms several roughly semicircular membranes or flaps which continuously change shape and position. The nucleus of this form does not differ from that of the ameboid phagocytes. Similar phagocytes have been described in earthworms where their membranes are oblong and arranged around the cell somewhat like petals.

Observations on freshly-drawn blood as well as on whole-blood preparations indicate that this form is very common in the perivisceral fluid. *In vitro* it was only found in a suspended condition and observations lead to the conclusion that it should be considered an adaptation to a floating way of existence, the membranes—apart from other functions—acting to increase the buoyancy, as in the case of planktonts. When the cell settles on a substrate it changes into an ameboid form, the membranes often persisting for a considerable time. A similar change was observed in the phagocytes of earthworms, when they come in contact with a solid surface (Liebman, unpublished observations) and in human leucocytes (Bessis and Bricka, 1949). There are indications that the ameboid form of Arbacia, if detached from the substrate, turns into the floating variety.

Common characteristics of the phagocytes. The nucleus is single, relatively large and never missing; its form more or less spherical, ellipsoid or bean-shaped. A large nucleolus, occasionally accompanied by one or two smaller ones, is always present.

The cytoplasm is hyaline and devoid of endogeneous inclusions. Ameboid movement appears in all forms in the presence of a substrate and increases markedly with the volume of the cytoplasm. Phagocytosis is present in all stages.

Function of the phagocytes. Though the phagocytes are known to be involved in several other functions, the present study is solely concerned with their capacity to take up formed matter. Under natural conditions this, except for occasional bacteria, consists almost exclusively of trephocytic granules or whole cells.

The flagellated forms were occasionally found to contain trephocyte granules of the green, red, or colorless variety in their cytoplasm. Because of their rapid movements, it could not be ascertained whether these are taken up by pseudopods or with the aid of the flagellum.

The ameboid forms phagocytose in the familiar way of engulfment with the aid of pseudopods. Generally, their phagocytic capacity appears to increase with the size of the cell or, more correctly, with the amount of the cytoplasm. In the fibroblasts, on the other hand, despite their relatively large size, the phagocytic capacity appears to be considerably reduced.

The petaloid forms show the highest phagocytic activity. This, however, is not necessarily due to an inherent quality but is probably owing to the ability to operate unimpeded in all three dimensions, in contrast to corresponding ameboid variants, phagocytosing on a substrate.

Whole, living trephocytes were repeatedly observed *in vitro* to be attacked by single, occasionally several, petaloid phagocytes. The trephocytes are caught by the flaps moving their surfaces close to each other and finally over the prey, somewhat in the manner in which a ball is caught in two hands. The engulfed trephocyte is seen moving vigorously within the phagocyte for a considerable time but ultimately undergoes gradual digestion, though its movements occasionally help it to "escape." This peculiar form of phagocytosis is also common within the organism, and whole-blood and *toto* preparations show a relatively high percentage of phagocytes containing intact trephocytes or some in various degrees of digestion (Fig. 3).

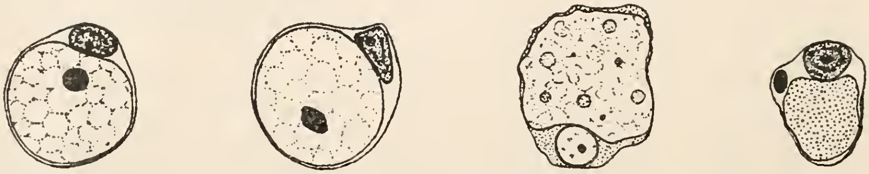


FIGURE 3. Phagocytes with engulfed intact and digested trephocytes; from whole-blood preparations; camera lucida; 1650 ×.

The uptake of trephocytic matter and the engulfment and digestion of living elements cannot be considered as phagocytosis in the accepted sense, which usually implies ingestion of noxious substances to be eliminated from the organism. It represents a normal way of nutrition for the phagocytes, and the phagocytosis of live trephocytes—cannibalism.[2] This, of course, should not imply that the phagocytes do not ingest matter destined for elimination, like dead cells, bacteria, or introduced foreign substances.

DISCUSSION

Previous studies on trephocytes (Liebman, 1946, 1947) have revealed that formed matter liberated by them is taken up by a variety of cells: oöcytes, phagocytes and epithelial cells. This led to the conclusion that, in addition to chemical substances, the animal cell depends also on biological elements for nutrition.

The present, as well as some earlier observations, impel us to extend this notion further, by putting forward the idea that cannibalism apparently represents a normal process of cellular nutrition.

Uptake and digestion of whole trephocytes by the growing ovarian eggs are apparently common among invertebrates. In some cases (*Tubularia*) the trephocytes taken up by the egg are dead, thus excluding cellular cannibalism as understood by cytologists. In several tunicates investigated, however, as well as in some annelids (Liebman, *ibid.*), the trephocytes within the egg appear to be alive. In the tunicates, they seem to persist in this state for a considerable time—as within the phagocytes of Arbacia—before being digested.

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[2] In the few instances where this term has been applied to cells, it is used both in a strict sense to denote ingestion of living elements by their own kind, as well as in cases where the phagocytosed cells are merely of the same orgasism. We propose to apply this term in the latter sense to all cases where a cell ingests and digests any other living cell of the same organism.

In the vertebrates, lymphocytes have been reported from epithelial cells of the intestinal and renal tract of fishes and Amphibia (Loreti, 1934, 1935), the oöcytes and epidermal cells of amphibians (Liebman, 1945b, 1947), and from the epithelial cells of the digestive and respiratory tract of mammals (Andrew and assoc., 1945, 1946, 1947). Not in all these cases are the lymphocytes alive when entering other elements nor are they all digested by the host cells, but there are clear indications that cannibalism in some of these instances is not infrequent.

It is possible that Cuenot (1891) saw elements corresponding to trephocytes within hyaline cells (phagocytes) but, failing to observe digestion, assumed that the former develop within the latter.

The presence of living cells within others has been definitely established in tissue cultures. Lewis (1925) saw frog-mononuclears and epitheloid cells from tubercular rabbits engulf their own kind, but stresses the great rarity of such cases and their probable pathologic nature. Fischer and Dolschansky (1929) repeatedly observed small cells wandering in and out of stroma cells of spleen cultures. They have not observed digestion of these elements, which they consider as derivatives of the stroma cells. Some of their photographs (e.g., Figs. 5, 6) indicate however, that these wandering cells are lymphocytes and their often fragmented and pycnotic condition does not exclude the possibility that they are occasionally digested by their hosts.

The inclusions of the trephocytes are frequently colored, occasionally also the cytoplasm. Except for echinochrome, however, our knowledge of their colorants is extremely limited and even the precise role of the former in respiration, is not fully clarified.

Observations on the invertebrate erythrocytes strongly suggest that they represent a special kind of colored trephocytes. It was pointed out earlier (Liebman, 1946) that the erythrocytes of the polychaete *Cirratulus grandis* contain numerous lipid inclusions in the cytoplasm and a typical trephocytic nucleus while their fixed shape, cytoplasmic structure and presence of hemoglobin are characteristic of erythrocytes. Conditions in other polychaetes (Romieu, 1923), as well as in invertebrates generally (Ohuye, 1937), reveal similar traits in their erythrocytes. This similarity extends to the vertebrates where the first embryonic (primitive) erythrocytes contain nutritive inclusions in the cytoplasm (Maximow, 1927), and often exhibit a striking resemblance to trephocytes (Cameron, 1941, and others). Loss of the nucleus, characteristic of the mammalian erythrocytes, occasionally takes place in invertebrate red corpuscles and is a recurrent feature of the trephocytes proper. It is significant that this trait is common only to the erythrocytes and trephocytes among the blood cells.

The presence of a flagellum in the youngest stages of the phagocytes is probably due to their origin from the coelomic epithelium which is usually ciliated in the echinoderms.

Petaloid phagocytes are quite common among invertebrates. Goodrich (1920) claims that the fine pseudopodes of the ameboid forms are either lateral views of the petals or artifacts, insisting that the only true form is the petaloid (his "membraneous") pseudopod. He also claims that the "petals" are merely folds of a continuous membrane. His extreme views are not borne out by our observations on Arbacia and earthworms.

Cuenot (1891) surmises that the movements of the flagellated forms prevent clotting of the blood. Coagulation of blood and the occlusion of wounds are generally agreed to be effected by the ameboid phagocytes forming a dense network with their fused pseudopods (pseudo-agglutination). Phagocytosis of introduced bacteria has been observed by Cernovodeanu and Henri (1906), and that of nondeposited sex products by Caullery and Siedlecki (1903).

SUMMARY

This investigation revealed the presence of two different types of leucocytes in Arbacia: trephocytes and phagocytes. An outline of their morphology, development and physiology is presented. No genetic relationship between the two kinds could be established, a circumstance which further tends to prove the distinctive nature of each type.

Particulate matter derived from the trephocytes and essentially of a nutritive nature, is taken up and incorporated by the growing oöcytes. A considerable fraction of this material has its origin in the red trephocytes which contain echinochrome. It is suggested that the echinochrome found in the eggs of the species investigated may also be derived from the trephocytes.

The occurrence of respiratory pigments among invertebrate trephocytes is discussed. It is pointed out that the erythrocytes of invertebrates, as well as the early stages of those of vertebrates, exhibit characteristics of trephocytes. The idea is advanced that the erythrocytes represent a special kind of trephocytes.

Formed trephocytic material was seen to be engulfed and digested by the phagocytes of Arbacia. This, as in the case of the oöcytes, is regarded as a normal process of cellular nutrition and lends further support to the thesis, expressed earlier by the writer, that the animal cell takes up and assimilates particulate cellular material in addition to substances in solution.

The engulfment and digestion of living trephocytes is described and discussed and additional cases of cellular cannibalism among invertebrates and vertebrates brought forward. The conclusion is reached that cannibalism is a normal and apparently fairly common form of nutrition of the animal cell.

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CONDITIONING V. MERCENARIA FOR SPAWNING IN WINTER AND BREEDING ITS LARVAE IN THE LABORATORY

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Rapid progress in studies of the morphology, physiology and ecological requirements of lamellibranch larvae, as well as other forms, can be achieved only if these organisms are easily available for study. This is seldom so in the case of lamellibranchs because the majority have a breeding period of comparatively brief duration lasting only a few months (Lebour, 1938; Thorson, 1946). During this period the larvae can be collected in plankton tows but, unfortunately, the early stages of many lamellibranchs are so alike in size and appearance that it is often impossible to distinguish with any degree of certainty the larvae of different species, or even genera. Thus, unless these larvae can be grown to metamorphosis their specific identity may remain in doubt. It is preferable, therefore, to work with larvae raised in the laboratory from fertilized eggs because in that case their identity cannot be questioned.

In obtaining material for morphological and physiological studies of larvae the advantages of the direct method, i.e., the method of raising larvae in the laboratory, over the indirect method of collecting them in plankton and often only guessing their identity, are clear. However, because lamellibranch larvae are small, and because their free-swimming period is relatively long, they are difficult to culture. As a result, with the exception of a few successful attempts, mostly confined to commercially important species, such as oysters and mussels, few lamellibranchs have been grown in laboratories past the early veliger stage (Thorson, 1946). Therefore, the need for a simple but reliable method for culturing them is, of course, obvious. It is hoped that the simple but efficient method described in this article for conditioning the hard shell clam, *V. mercenaria,* to spawn out of season and for culturing its larvae will be applicable to many other species of lamellibranchs permitting their successful cultivation in the laboratory, where their morphological features and various aspects of behavior can be studied under controlled conditions.

Realizing the importance of having a good supply of larvae for studies of the life history of *Venus mercenaria,* Belding (1912) tried to raise them in the laboratory. He was not successful because most of the larvae in his cultures died either before they reached the straight hinge veliger stage or soon afterwards. Belding concluded that there was no practical method for raising hard shell clams to the setting stage because of the small size and delicate nature of the eggs. Several years later, nevertheless, Wells (1927) succeeded in growing clam larvae to the setting stage. Wells, however, was mostly interested in oysters and did not continue the clam work.

Obtaining clam spawn in the summer time is relatively a simple procedure. During the summers of 1933, 1934 and 1935 many clams were induced to spawn simply by raising the water temperature a few degrees (Loosanoff, 1937). However, because the spawning season of *V. mercenaria* in our waters is confined to a period of approximately 2½ or 3 months, experimenting with their eggs and larvae was necessarily also confined to the same period of time. Fortunately, since a method has been recently devised to induce oysters to develop spawn in the winter time (Loosanoff, 1945), it was decided to apply this method to clams also, hoping in this way to extend considerably the period during which ripe eggs and sperm could become available for laboratory work. The method was successful, and it is now possible to make clams spawn, releasing normal gametes, throughout the winter and spring.

The method for conditioning clams for spawning in the winter time is as follows: Clams brought from their natural beds in Long Island Sound, where the temperature of the water in the winter time is near 0.0° C., are placed in trays of running sea water having a temperature of approximately 5.0 to 7.0° C. Then, at intervals of 3 to 5 days the temperature of the water is increased by several degrees. Eventually the temperature is raised to about 20.0–22.0° C. and the clams soon become ready for spawning. The entire conditioning period usually takes about 3 weeks, but can be made even shorter, if the intervals between the increases in temperature are shortened to about one day. The clams may also be placed directly into water of a temperature of about 20.0° C., but under such a condition some mortality may occur.

Conditioned clams were induced to spawn by raising the temperature several degrees above the conditioning level. However, if the temperature was raised above 34.0° C., most of the clams usually withdrew the siphons and closed the shells. It was often noticed that spawning began during a decrease in temperature, i.e., if the temperature was first raised to about 35.0° C. and then gradually decreased.

In several instances cases of spontaneous and apparently unprovoked spawning were observed at temperatures several degrees lower than 23.0° C., which had been considered the minimum at which clams could spawn (Belding, 1912; Nelson, 1928; Loosanoff, 1937). For example, on February 7 and March 4, 1949, clams were seen spawning at 22.0° C., and on March 28 and April 5 large groups spawned in the trays having a temperature of only 21.0 and 20.6° C. respectively. In all cases both males and females were spawning, many of them quite profusely. The eggs from these spawnings were collected and cultured, the larvae reaching the setting stage.

An individual clam does not discharge all its eggs or sperm at one spawning but continues to spawn, at intervals of a few days, over an extended period. For example, in one of the groups a marked female was induced to spawn on six different occasions between February 2 and March 3. Many other clams of the same group also spawned several times. In general, this group provided spawn for a period of approximately 5 or 6 weeks, before the majority of the clams became spent.

Not all the eggs discharged by the spawning females possessed the same vitality. Probably some clams were compelled by the strong temperature stimulation to abort the eggs even if the eggs were not fully ripe. Such eggs usually developed into feeble larvae which soon died. The last batches of eggs discharged by virtually spent females also gave feeble larvae that grew slowly and showed a high mortality. With a little experience, however, an investigator can learn to recognize various types of spawnings and select only those batches of eggs that are suitable for cultivation.

The eggs to be used for cultivation of the larvae were fertilized as soon as they were discharged. To separate them from the debris accumulating in the spawning dishes the water containing the eggs was run through a Number 80 stainless steel testing sieve, having openings of 177 microns. These openings were large enough to let the eggs surrounded by membranes pass through but retained the larger particles. After that the egg suspension was filtered once more through another sieve, which was fine enough to retain the eggs but let the water containing the sperm, blood cells, etc., pass through. The retained eggs, now free of all impurities, were placed in fresh sea water in glass hatching jars of about 14 liters' capacity, which were continuously aerated.

The eggs and later young larvae remained unattended until they developed into early veliger. Then the water in the jars was renewed every second day. To accomplish this the content of the jars was strained through fine sieves, which retained the larvae but let the water pass through. The jars were then filled with new water and the larvae returned to them.

To feed the larvae, small quantities of mixed plankton cultures, consisting primarily of forms of less than 5 microns in size, were added daily to each jar. The food cultures were grown during the winter in large aquaria, and in the summer in the outdoor, concrete tanks filled with sea water and enriched with a commercial fertilizer (Loosanoff and Engle, 1942). Within a few days extremely rich, mixed cultures consisting chiefly of phytoplankton were available for feeding the larvae of the clams. This food was also used to feed numerous cultures of larvae of two other species of clams, *Mya arenaria* and *Mactra solidissima*, and three species of oysters, *Ostrea virginica, O. gigas,* and *O. lurida,* which were grown to metamorphosis at our laboratory.

When the larvae were reaching the setting stage old oyster shells were placed on the bottom of the jars to provide a place for attachment, or the larvae were transferred to special aquaria on the bottom of which a layer of sand was spread.

A description of the development of the clam egg and of the morphology of larvae has already been given by Belding (1912), on the basis of material which he found in plankton. Therefore, it is not necessary here to go into most of the details. Instead we shall offer a brief account of the clam development from fertilized eggs to the dissoconch stage, as observed under laboratory conditions at a temperature of about 22.0° C.

The egg of the clam measures from about 70 to 73 microns in diameter (Fig. 1, A). It differs from the eggs of some other lamellibranchs in that it is surrounded by a thick gelatinous membrane the diameter of which usually is within the range from 163 to 170 μ. It was noticed on many occasions that this membrane continues to surround the egg past the blastula stage and, sometimes, until trochophore larvae are formed. If at fertilization spermatozoa are numerous, many can be seen imbedded in the outer portion of this membrane (Fig. 1, A).

The fertilized egg will reach the two-celled stage in about 45 minutes (Fig. 1, B), and the four-celled stage is reached in about an hour and a half (Fig. 1, C). In about 6 hours the embryo becomes a well-developed, rotating, ciliated blastula. The early gastrula stage is reached after 9 hours, and finally the larva enters into the trochophore stage which is reached about 12 hours after fertilization (Fig. 1, D). This form, roughly pear-shaped, moves through the water with a spiral motion,

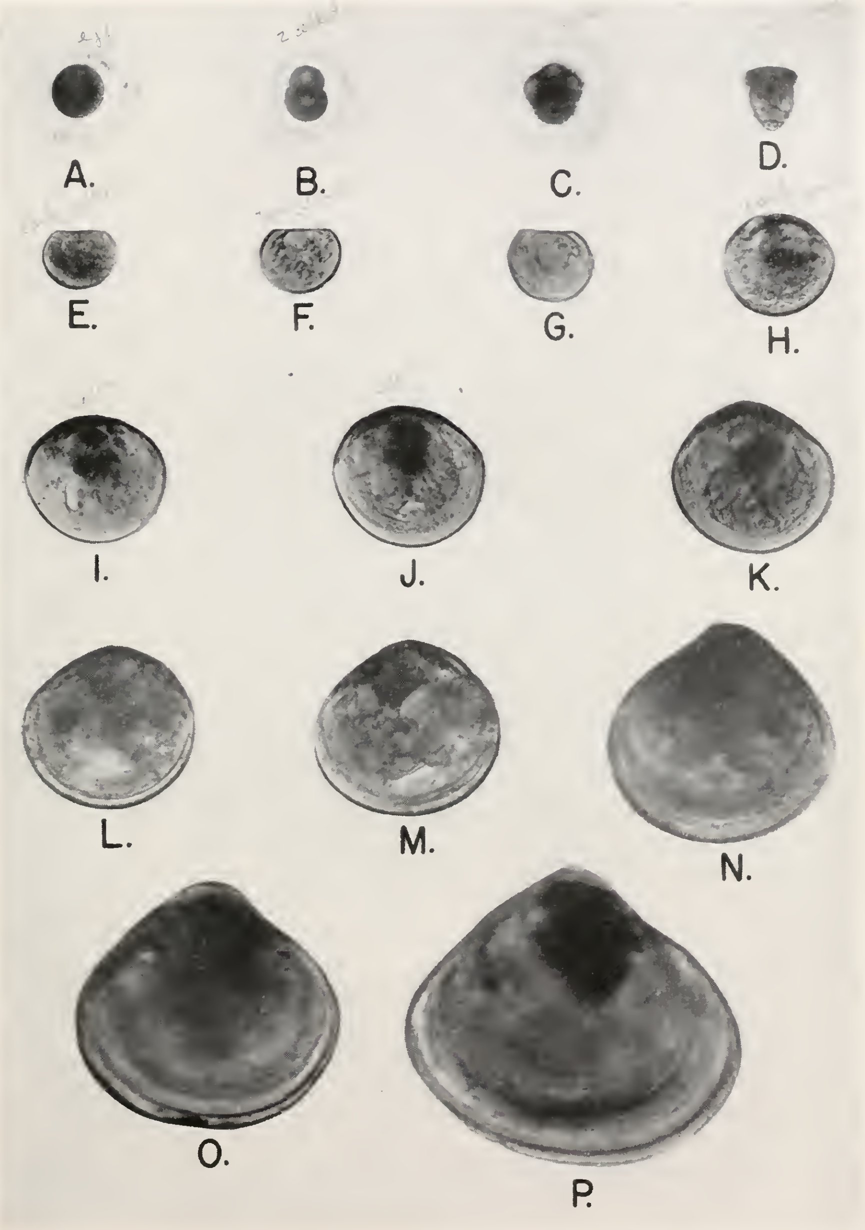
egg
2 cell
A.
B.
C.
D.
E.
F.
G.
H.
I.
J.
K.
L.
M.
N.
O.
P.

FIGURE 1. Different stages of development of clams through larval and early post-larval stages. × 112. Description in text.

propelling itself by a circlet of cilia around the anterior end, aided by a velar tuft of longer cilia at the extreme anterior end. In this respect it differs from the gastrula stage during which almost the entire body of the larva was covered with small cilia. Furthermore, the trochophore larva begins to form a primitive mouth and develops a shell gland. The larva at this time measures about 90 × 65 μ.

As the development progresses, a small thin shell is secreted by the shell gland and is gradually extended to cover the entire animal. This usually occurs from 24 to 36 hours after fertilization and the larva is now in the early veliger or early straight hinge stage (Fig. 1, E). Its size at this time is approximately 105 × 80 μ. About 8 to 12 hours later a true veliger or straight hinge stage is attained (Fig. 1, F). At this stage the larva, which is approximately 110 × 90 μ in size, becomes quite a proficient swimmer using for this purpose its highly developed velum.

If conditions are favorable, the veliger continues to grow reaching the size of about 122 × 98 μ by the end of the fourth day, but still remaining in the straight hinge stage (Fig. 1, G). By the sixth day it is already in the early umbo stage and measures approximately 154 × 143 μ (Fig. 1, H). In 8 days some rapidly growing larvae may reach the size of 205 μ, while the average are in the medium umbo stage measuring 195 × 178 μ (Fig. 1, I). About 10 days after fertilization many individuals in good cultures are in the late umbo stage, measuring about 214 × 192 μ (Fig. 1, J), and after 12 days some of the mature, ready-to-set larvae may be as large as 227 ×210 μ (Fig. 1, K).

Just prior to this stage clam larvae begin to undergo prominent changes. The velum begins to disappear and some of its functions are taken over by a foot which is covered with numerous cilia. At first this ciliated foot aids in swimming, but gradually is used more in gliding over the bottom and in crawling. Eventually the velum entirely disappears, thus ending the free-swimming period.

In most cultures many larvae reached the setting stage in about 12 days. However, in some cultures grown at a temperature of about 24.0° C. metamorphosis occurred in ten days. The smallest metamorphosed clams were only about 210 microns. They were often seen attached by the byssus to the shells which were placed on the bottom of the aquaria. Thorson (1946) states that the veligers of *Venus gallina* also very often set when they are only about 210 μ although the length of the prodissoconch varies between 210 and 225 μ. We found even greater variations in the size of the prodissoconch shell of *Venus mercenaria* because some of them were as large as 240 μ. Nevertheless, none of the prodissoconch of our cultures ever approached the size of 320 μ as reported by Sullivan (1948) for the Venus larvae of Malpeque Bay. However, as Jørgensen (1946) showed, the size of the larvae at the time of setting may vary considerably according to the conditions of the environment and, therefore, the measurements made at setting are only of relative importance.

After metamorphosis the young clam begins to form the adult or dissoconch shell. Successive stages of growth showing the increase in size and formation of adult shell are given in Figure 1, L, M, N, O and P. The sizes of these small clams were 240 × 223, 260 × 245, 313 × 308, 366 × 340 and 463 × 423 μ respectively. The oldest individual shown was 28 days counting from the day of fertilization.

Significant variations in the sizes of the individual larvae of the same cultures were very often noticed. For example, while some of the largest larvae were approximately 210 μ in size and were ready to metamorphose, the other larvae of the same culture were only 150 or 160 μ long and were still far from the end of the free-swimming stage. Sometimes, because of overcrowding, a difference in temperature or other factors, the average size of the larvae of two parallel cultures carried in separate jars would also show significant differences.

Clam larvae were not too selective in their food, surviving and growing rapidly on many micro-organisms instead of being confined to a few forms. The exception was when the clam larvae were fed almost a pure culture of a certain species of Chlorella. The larvae so fed grew more slowly and showed a heavier mortality than those which were fed mixed plankton cultures.

We found that the method for staining oyster larvae, which we described some time ago (Loosanoff and Davis, 1947) is also applicable to clam larvae. By using a weak solution of Neutral Red, clam larvae were stained and thus became easily distinguishable from the normal individuals. It is believed that this method will help, later on, to study the dispersal of larvae from the place of origin, their rate of growth under natural conditions, etc.

In conclusion it may be said that by following the few simple principles and rules given in this article mature sperm and eggs of *V. mercenaria* and several other species can now be obtained on almost a year-round basis, and the larvae can be grown to the setting stage even in the middle of winter.

We wish to express our thanks to our colleague, Mr. Charles A. Nomejko, for making the microphotographs of the larvae for this article.

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THE CONTROL OF RETINAL PIGMENT MIGRATION IN LEANDER SERRATUS

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Introduction

The movements of pigments in the eyes of crustaceans are known to be related to the illumination of the eye and to the shade of the background on which the animals are placed, but the means by which these pigment movements are controlled have been disputed for more than fifty years. Conflicting evidence has pointed on the one hand to a hormonal control of these pigment movements and on the other hand to a direct response of the pigment cells to the presence or the absence of illumination. There is also the possibility that the pigment movements are under direct nervous control, but as yet no nervous connections to the distal or the reflecting pigment cells have been traced, and the proximal retinular cells containing pigment are innervated by sensory fibres only (Parker, 1891). Recent evidence has indicated that the retinal pigment cells respond to the injection of hormones, but this does not exclude the possibility that they may also respond directly to illumination, as some of the earlier evidence indicated. The present paper is an attempt to evaluate the relative importance of hormonal control and a direct response to illumination in movements of the retinal pigments in the prawn *Leander serratus*.

In 1897 Parker described experiments on *Palaemonetes vulgaris*, in which one eye was covered and the other was exposed to direct illumination. In one experiment, one eye was covered by a mixture of Canada balsam and lampblack; another experiment consisted in placing an animal in a light-proof box in such a way that one eye projected through a hole in the side of the box and was exposed to illumination. Parker reported that, in his experiments, "the eyes exposed to the light always presented the condition normal for light, and those kept in the dark always showed an approach, more or less complete, to the condition characteristic for the dark."

Experiments comparable to those of Parker were carried out by Castle (1927), who used plaster of Paris to cover the eyes of Palaemonetes and studied the effects on the movements of the proximal pigment only. Covering of one eye or of both eyes caused complete or incomplete darkness-adaption of the covered eye, although when one eye was covered and the other was exposed, the latter always showed the condition characteristic of light-adaption.

Further experiments were carried out by Bennitt (1932) on *Palaemonetes vulgaris* and *P. exilipes*, using Castle's method of covering the eye. One third of the covered eyes showed a partial adaption to darkness, but only one in forty-five of the exposed eyes of the same animals showed an intermediate state—the rest were light-adapted.

Bennitt then explored the possibility that light might be entering the covered eye through the transparent eye-stalks. To avoid this difficulty he used opaque animals, namely Cambarus, Cancer, Carcinides, and Libinia. The results, however, were inconclusive; in some cases the illuminated eye was light-adapted and the covered eye was partially dark-adapted, while in other instances both eyes became light-adapted. Bennitt concluded that pigment movements in the two eyes of a crustacean were interrelated and that stimulation of one eye would bring about pigment migrations in both eyes; he suggested that the two eyes might be interrelated through hormonal control.

The retinal pigments of many species of crustaceans exhibit a marked diurnal rhythm of migration; in some species a diurnal rhythm persists in constant light, and in others it continues in constant darkness. The three retinal pigments behave differently in the various species. The evidence has been summarized by Brown (1944) and by Kleinholz (1949*a*). The existence of these diurnal rhythms reveals that factors other than direct illumination cause the retinal pigments to migrate. In 1930 Welsh showed that when a dark-adapted Palaemonetes was placed in the light for twenty minutes and then returned to darkness, the migrations of the retinal pigment, which began under illumination, continued for the next twenty minutes in darkness, although the stimulus for migration was no longer present. These observations led Kleinholz (1936) to investigate a possible hormonal control of distal pigment movements in Palaemonetes. His injections of eye-stalk extracts into dark-adapted animals brought about the light-adapted position of the distal and the reflecting pigments, the proximal pigment remaining unaffected by the injected extract. Welsh (1939) has subsequently shown that injections of concentrated eye-stalk extracts will cause the proximal pigment of dark-adapted Cambarus to move into the position characteristically found in light-adapted animals. It has not yet been shown that in any one species the distal, reflecting and proximal pigments can all be moved by injections of hormones, and in some species one or another of these pigments remains in a fixed position, and is unaffected by injections or by change of illumination. It seems clear, however, that the three pigments of the crustacean eye will respond to injections of eye-stalk extracts, although these injection experiments alone do not determine that the control of the pigment movements is predominantly hormonal under normal conditions. The experiments described above, which indicated a partial independence of the covered and the exposed eye of an individual, showed that other factors besides hormonal ones were involved. In the light of so much confusing evidence it seemed desirable to investigate the matter, using *Leander serratus* as a test animal.

Part of the present work was done at Plymouth and the remainder at Naples. I should like to acknowledge my gratitude to the Challenger Society which defrayed many of my expenses at Naples, and to Professor A. C. Hardy, F. R. S., who kindly allowed me to use the Oxford table there. On one occasion I occupied the table at Plymouth rented by the British Association and on another occasion that rented by the Clothworker Company. I am greatly indebted to these two bodies for the use of their tables at Plymouth. I am also indebted to the Directors and staffs of the Laboratories of the Marine Biological Association at Plymouth, and the Stazione Zoologica at Naples for their unfailing courtesy and help.

MATERIAL AND METHODS

In the present experiments large male individuals of *Leander serratus* were used. In order to have some means whereby the positions of the distal retinal pigment might be expressed numerically and compared in eyes of different size, the following procedure was adopted. Measurements were taken of the distance from the basement membrane to the distal ends of the distal retinal pigment cells, and also from the basement membrane to the surface of the eye (see text Fig. 1). The position of the distal pigment can thus be expressed numerically as the ratio of the former measurement to the latter, and is denoted in the present paper as the distal retinal pigment index; this measurement provides a satisfactory method of comparing the response of the distal retinal pigment in eyes of different size.

In those experiments which required an opaque cover for the retina some difficulty was experienced in finding a satisfactory substance; one which would not dissolve in sea-water and yet did not contain a solvent that would affect the retina. In a preliminary series of experiments the mixture of Canada Balsam and lampblack used by Parker (1897) was employed, but it was found that the xylene solvent itself caused positional changes in the retinal pigments and the use of this mixture was discontinued. Eventually red modelling clay (marketed under the name of Iwana) was found to be satisfactory for covering the eyes. In applying this clay it was found convenient to secure an animal by fixing two tapes over the body, one over the cephalothorax and the other over the abdomen, and pinning these to a small board. A bit of modelling clay was fashioned into a ring which was slipped over one eye and attached firmly around the exoskeletal base of the eye-stalk; a second ring was then slipped over the eye-stalk and by using the fine point of a needle was joined to the first ring; finally, a small cap of clay was fitted over the retinal portion of the eye and moulded to the second ring around the eye-stalk. In this way a loose opaque sac of modelling clay was fitted over the eye and its stalk. The smaller diameter of the eye-stalk prevented the cover from falling off.

An alternative method of covering one eye is the box method described by Parker (1897). The animal is fastened by tapes to the inside of a light-proof box in such a way that one eye-stalk projects through a hole just wide enough to admit it. The box is then placed under water in a darkroom with a beam of light directed on the exposed eye along a plane parallel to the side of the box; this direction is calculated to allow the least possible amount of light into the interior of the box. The box experiment and the method of covering the eye with clay provide two contrasts in method. In the box method the covered eye is not constrained in any way, whereas in the other experiment it bears a clay covering. In the clay experiment the body is illuminated but in the box experiment it is not.

The intensity of light in these experiments was measured by a Weston photometer, which expressed light-intensity in terms of seconds necessary for photographic exposure. This did not give an accurate absolute measurement but was sufficiently sensitive to permit an adjustment of the source of illumination so that approximately the same intensity of light fell on the eye in each separate experiment. The temperature of the water was taken before and after each experiment. In no case had the temperature risen more than 2.0° C. at the end of a four-hour interval. Black, white and grey backgrounds consisting of painted cake tins were used in some of the experiments.

Removal of the sinus-glands in some of the experiments was accomplished by a modification of the method described by Panouse (1946). The animal to be operated upon was secured by tapes as described above, a piece of modelling wax was arranged at one side of the head and a groove was made in the wax in which one eye could rest. The operations were generally performed in the evening after the animals had been in darkness for some hours so that the contraction of the chromatophores on the eye-stalk allowed the sinus-gland to be seen clearly through the cuticle of the stalk. The cuticle was lightly pierced above the gland with the points of fine watchmaker's forceps, and a small piece of the cuticle was removed; the aperture made was sufficiently large to allow the points of the forceps to pass through and seize the sinus-gland which is a bluish-white mass in contrast to the more translucent surrounding tissues (Fig. 2). After removal of the sinus-gland the hole in the cuticle was filled by a small plug of modelling clay which prevented any further loss of blood. In each animal excision of the second sinus-gland was performed 24 hours after removal of the first gland.

The success of sinus-gland removal was judged by (1) direct observation of the eye-stalk, (2) the behaviour of the dark pigments in the chromatophores, (3) sectioning the eye-stalks after experiment. Immobility of the dark chromatophoral pigments for ten days, under various conditions of illumination and background, was taken as an indication of successful sinus-gland removal. Later sectioning of the eye-stalks confirmed this opinion. About one-third of the operations were successful, a figure which is in agreement with those obtained by Panouse (1946) and Brown, Ederstrom and Scudamore (1939). The mortality among operated animals was about 25 per cent. In all, 148 experimental and 18 control animals were examined.

A preliminary series of experiments revealed a diurnal rhythm of activity of the proximal and reflecting pigments (Fig. 3). In constant darkness, these pigments became more completely dark-adapted at midnight than they did at midday. To avoid the extremes of this rhythm, all subsequent experiments were carried out during the afternoon and in no cases finished later than 6 p.m.

After each experiment the animals were immersed in hot water (80° C.) for 10 seconds to fix the position of the retinal pigments. The eyes were excised and transferred to a modified Bouin's solution (containing 7 per cent acetic acid) for 24 hours. They were then placed successively in 70 per cent alcohol for 3 hours, in dioxane for 12 hours and in paraffin wax for 3–5 hours. Before the final embedding, the eyes were oriented in the melted paraffin with the dorsal side uppermost (readily recognized by the black pigment-spot located at the junction of the retinal and non-retinal portions of the eye-stalk). Serial sections were cut at 15–20 μ and were generally mounted unstained for observation of the position of the retinal pigments. Measurements of the position of the distal pigment were made on the central ommatidia of those sections bearing the dorsal pigment spot; thus the measurements represent, as nearly as possible, the position of the distal pigment cells in the central ommatidia of the eye.

THE EYE OF LEANDER SERRATUS

The structure of the decapod eye has been described in detail by many authors: a survey of the literature on the subject has been given by Parker (1932) and a more recent account of the structure of the eyes of some species has been given by Debaisieux (1944). There is, however, a lack of uniformity in the terminology used by different authors to describe corresponding parts of the eye in different species.

The eye of *Leander serratus* is composed of about 7000 ommatidia and a dorsal accessory pigment spot. Each ommatidium (Fig. 1) comprises five cell-types as

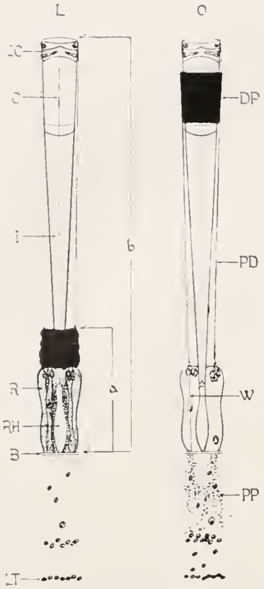
L
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FIGURE 1.

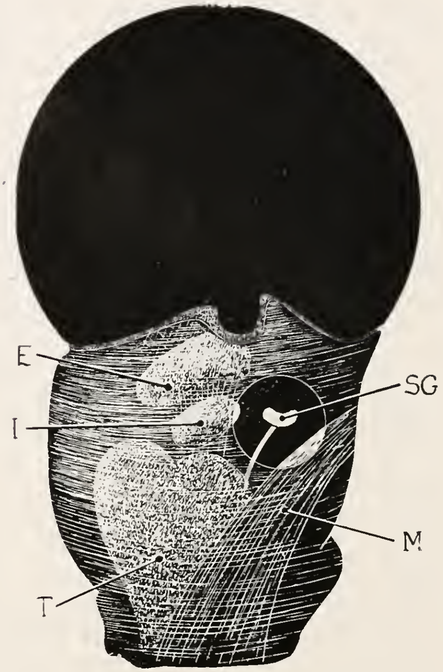

FIGURE 2.

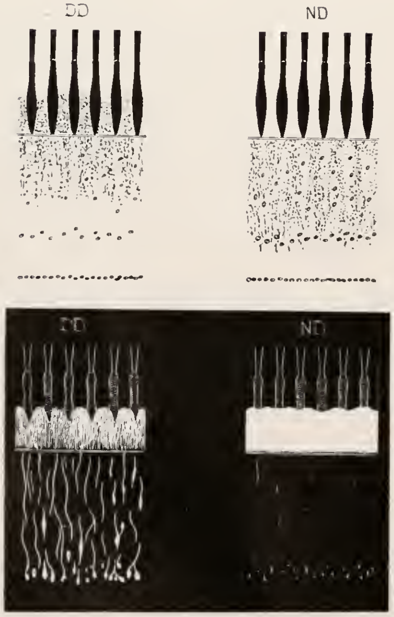

FIGURE 3.

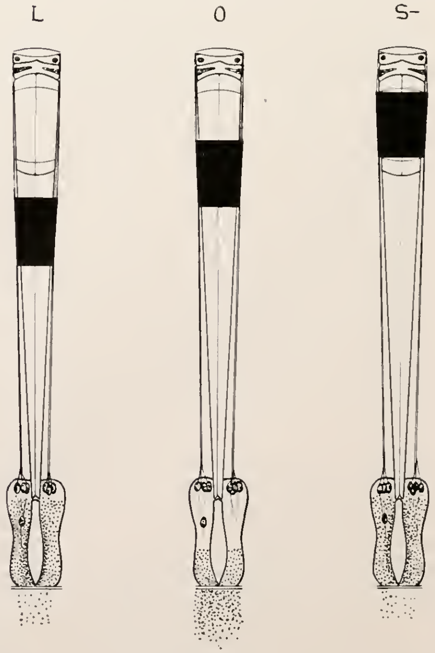

FIGURE 4.

follows: (a) Corneal hypodermal cells which secrete the cornea. (b) Cone cells which form a crystalline cone. (c) Distal retinal pigment cells. (d) Proximal pigmented retinular cells, which surround a central transparent mass, the rhabdome. (e) Tapetal pigment cells, of which there is one cell to a number of ommatidia.

The cornea is facetted in such a way that each ommatidium bears, at its distal end, one facet which is square in surface view and slightly biconvex when viewed in section. Each facet is composed of a thin scaly epicuticle and a thicker cuticle. Two corneal hypodermal cells lie beneath each facet.

The cone consists of four elements which form an elongated pyramid with its square base approximated to the proximal surfaces of the corneal hypodermal cells. In this basal portion there are four nuclei, corresponding to the four elements of the cone. Each cone element is not homogeneous throughout its length, but comprises a cell-body, the crystalline cone proper, and an extension which Debaisieux (1944) calls an intermediate crystalline tract. Distally, prolongations of the cone cell-bodies appear to pass between the corneal hypodermal cells and may come in contact with the corneal facet. Proximally the intermediate crystalline tract is prolonged into a number (Debaisieux states that there are 11 to the four crystalline tracts in *Palaemon varians*) of fine fibres which extend proximally between the retinular cells and envelop the rhabdome; in some species these fibres extend to the basement membrane, but in Leander neither Schneider (1902) nor Trojan (1913) could trace them as far as this.

The cone is surrounded by two pigment cells which have been known variously as "Hauptpigmentzellen," "Iris pigment cells," "Distal retinular cells," and "Pigmentzellen." The name "Distal pigment cell" has been widely adopted in recent literature and will be used in the present account. These distal cells contain mainly dark pigment granules, but in addition there is a thin covering sheath of white light-reflecting pigment. The distal and the proximal extensions of the distal pigment

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FIGURES 1–4.

FIGURE 1. Light adaptation in the eye of *Leander serratus*. Two ommatidia are shown, the left in a condition of total light-adaptation, the right with the pigments in a dark-adapted position. The change in position of the distal and proximal pigments is depicted; the position of the reflecting pigment is not shown. The measurement a/b is described in the text as the distal pigment index.

CC. Cells of crystalline cone. C. Cone. I. Intermediate crystalline tract. R. Retinular cell. RH. Rhabdom. B. Basement membrane. LT. Lamina ganglionaris. DP. Distal pigment. PD. Proximal extension of distal pigment cell. W. Distal extension of reflecting pigment cell. PP. Proximal pigment.

FIGURE 2. The position of the sinus-gland in the eye-stalk of *Leander serratus*. The right eye is viewed from above, and a small aperture has been cut in the cuticle directly above the sinus-gland. M. Muscle. SG. Sinus-gland. E. Medulla externa. I. Medulla interna. T. Medulla terminalis.

FIGURE 3. Diurnal rhythm of proximal and reflecting pigments in *Leander serratus*. The movements of the proximal pigment are shown in the upper drawings; the reflecting pigment is depicted in the lower figures. On the left, the retina of an individual killed at midday after 24 hours in constant darkness (DD). On the right, the retina of an animal killed at midnight after 12 hours in constant darkness (ND).

FIGURE 4. Illumination and the sinus-gland hormone in the movements of the distal pigment in *Leander serratus*. L and O are ommatidia from the exposed left and covered right eye respectively of an animal maintained in a box with one eye projecting through a hole in the side of the box. S- is an ommatidium from an individual from which the sinus-gland had been removed a week previously, and which had been for four hours exposed to powerful illumination.

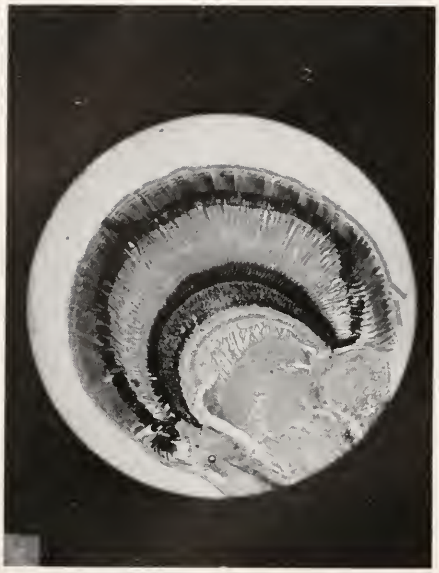


Figure 5.

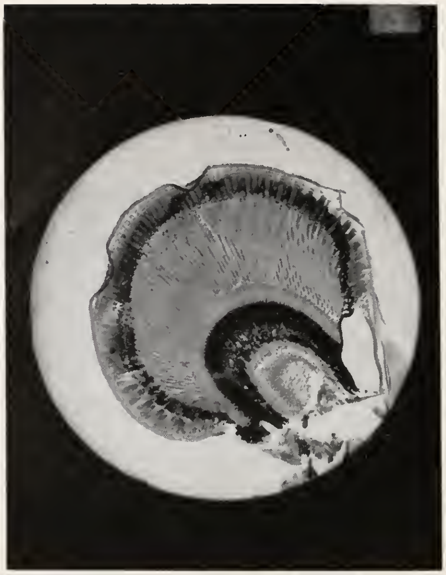
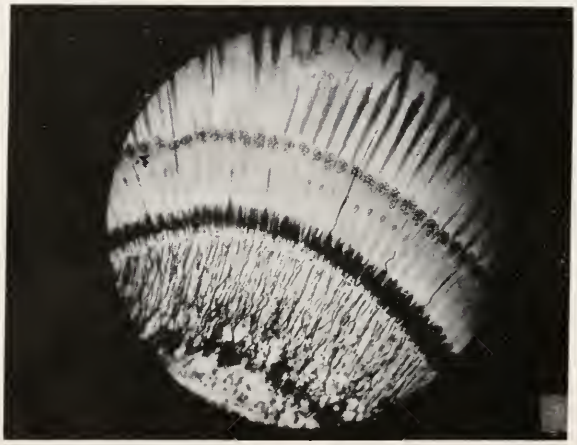


Figure 6.

Figure 7.



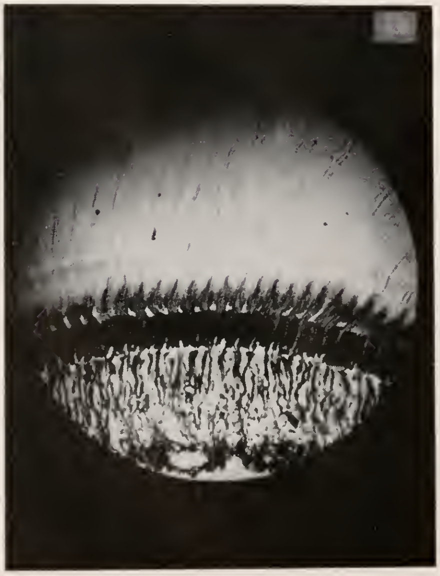


Figure 8.

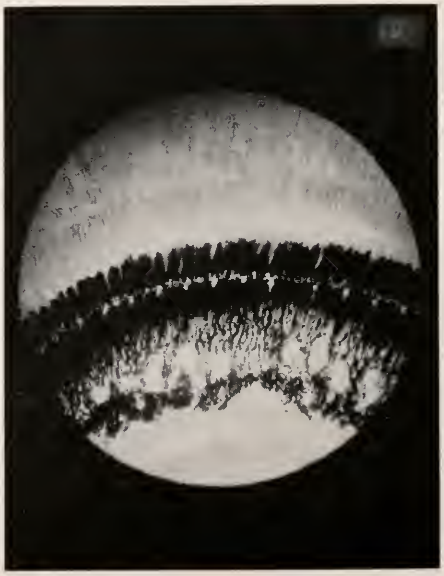


Figure 9.

cells have been variously described in the literature. Welsh (1930) depicted a distal extension of the pigment cells to the cornea in *Palaemonetes vulgaris* and a proximal extension by fibres to the distal ends of the retinular cells. Debaisieux (1944) shows a distal extension to form a collar around the corneal hypodermal cells and proximal fibres extending to the basement membrane. In eyes containing both dark and white pigments the connections are not easily distinguished because the white pigment appears dark by transmitted light and, moreover, it is partly masked by the dark pigment. In my preparations of retinas of *Leander serratus* I have observed that if the dark pigment is removed by controlled immersion in dilute ammonia, but the white pigment remains, the extensions described by Debaisieux can be seen (Fig. 7). If the white pigment only is removed, by immersion in boiling water, the connections depicted by Welsh can be observed. I believe that the inner darkly pigmented portion of the distal cell is in continuity with the retinular cells proximally and the cornea distally, but the sheath of white pigment is in continuity with the white pigment of the tapetal layer proximally and the cornea hypodermal cells distally.

There are eight proximal retinular cells, though one of these is very reduced in size. Proximally these cells taper to nerve fibres which pass through the basement membrane and gather to form the lamina ganglionaris, whence fibres pass to the medulla externa of the eye-stalk. The extensions of the retinular cells between the basement membrane and the lamina ganglionaris are fairly wide, and pigment contained within the retinular cells migrates above or below the basement membrane in response to the illumination falling on the eye. The pigment contained in the retinular cells is known as the proximal pigment.

The tapetal layer comprises a number of nucleated cells, about one to each ten ommatidia, which lie distally to the basement membrane, but with processes extending proximally towards the lamina ganglionaris. The tapetal cells contain a white light-reflecting pigment, and have been compared by Debaisieux to the white chromatophores of the integument; distally they extend to the distal pigment cells and beyond. The pigment they contain is referred to as the reflecting pigment in this account.

Movements of the Retinal Pigments Under Normal Conditions of Illumination

To study first the normal migrations of the retinal pigments, on illuminated backgrounds and in darkness, the following procedure was adopted: Ten large male individuals of *Leander serratus* were placed in a dish on a white background and ten

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Figures 5–9.

Figure 5. The exposed and illuminated right eye of an individual maintained in a light-proof box for three hours.

Figure 6. The covered left eye of the same individual shown in Figure 5.

Figure 7. A small portion of the retina of *Leander serratus* from which the dark proximal pigment has been removed. The white reflecting pigment appears dark by transmitted light. The distal extensions of the reflecting pigment cells are shown.

Figure 8. The retina of the exposed and illuminated eye of an individual kept in a light-proof box for three hours. The proximal pigment is in the extreme light-adapted position.

Figure 9. The retina of the darkened eye of the same individual as Figure 8. The proximal pigment is in the position of greatest dark-adaptation found in any animal killed during the day.

individuals were similarly placed on a black background; both sets of individuals were exposed to the same intensity of incident illumination. A third group of ten individuals was placed in darkness. The experiment was begun at 2 p.m. and continued until 6 p.m. when the animals were killed. Welsh (1930) reported that the average time required in *Palaemonetes vulgaris* for the distal pigment of a dark-adapted individual to become completely light adapted in the light was approximately 40 minutes, while about 90 minutes was required for the reverse process of dark-adaption. Migration of the proximal pigment is more rapid than that of the distal pigment. Although observers have given different times for the various species of decapods studied (Parker 1932), in no case do these estimates exceed 2 hours. The results of illumination and background experiments on the migration of the distal pigment are given in Table I.

TABLE I

The position of the distal retinal pigment under various conditions of illumination and background. Each experiment was carried out on ten animals. I, is the intensity of incident light measured in terms of seconds necessary for photographic exposure (see methods); R, is the intensity of reflected light. The standard deviation is calculated by the formula $S.D. = \sqrt{\frac{\Sigma D^2}{n}}$.

Light intensity			Nature of background	Average distal pigment index and standard deviation
I	R	$\frac{I}{R} \times 1000$		
.04	.33	120	white	.35±.067
.04	8	5	black	.41±.01
—	—	—	darkness	.75±.006

The distal pigment *indices*, for a second series of experiments carried out under similar conditions, ranged from .27–.3 for a white background, from .5–.6 for a black background and from .75–.90 in darkness. To test the value of this distal pigment index for comparing the position of the distal pigment in different species, the figures given by Kleinholz and Knowles (1938) were calculated in the same way. Treated thus, they gave a range of from .31 to .60 for eyes of animals placed on backgrounds ranging from white to black, and exposed to such conditions of illumination that the ratio, $\frac{I}{R} \times 1000$, ranged from 9 to 62. The figure for darkness was .76. It will be seen that these figures obtained in *Leander adspersus* correspond closely to those obtained in the present experiments on *Leander serratus*. It is thus evident that in these species the distal retinal pigment migrates distally in darkness and proximally under illumination and that the shade of the background influences the position of the distal pigment.

On the other hand, the proximal pigment in the retinular cells did not show differences in animals which had been on different backgrounds under illumination, though this pigment did, however, show differences between illuminated animals and those maintained in darkness. Under illumination the proximal pigment migrated distally so that it came to lie above the basement membrane, extending along the rhabdom as far as the nuclei of the retinular cells; in darkness a great part of the proximal pigment migrated below the basement membrane and lay between it and the lamina ganglionaris.

Persisting Diurnal Rhythm

The retinal pigments of many crustaceans show a marked diurnal rhythm of migration, which may continue in constant light or in constant darkness in different species. The literature has been reviewed by Kleinholz (1942). Inasmuch as Panouse (1946) had reported that chromatophoral pigments of *Leander serratus* migrated diurnally in constant darkness, it seemed advisable to determine whether a persisting diurnal rhythm of the retinal pigments was present in this species. Fifteen individuals were taken from a tank and placed in darkness at 9 a.m. one day. At 12 noon on that day five of these individuals were killed, and the water changed for the others; at midnight the following night five more individuals were killed and the water once more replenished; at midday the following morning the remaining animals were killed. The eyes were compared after sectioning. No significant differences in the position of the distal pigment could be observed, but the positions of the reflecting and of the proximal pigments differed in animals which had been killed at midday or at midnight (Fig. 3). The reflecting pigment of animals killed at night had migrated distally to the basement membrane and lay between the retinular cells; on the other hand, in individuals kept in darkness and killed at midday, the reflecting pigment was dispered above and below the basement membrane. A similar condition was observed in illuminated animals killed at midday. The proximal pigment of Leander in darkness killed at night had migrated proximally to the basement membrane and lay between it and the lamina ganglionaris; while the proximal pigment of animals maintained in darkness and killed at midday lay partly below and partly above the basement membrane. It did not extend distally beyond the tapetal layer (about half the distance along the length of the rhabdome). In view of this persisting diurnal rhythm of proximal and reflecting pigment movements, all experiments were conducted at the same time each day.

The effect of covering one eye

In a preliminary series of experiments carried out using Parker's (1897) method of covering an eye with a mixture of Canada balsam and lampblack, animals thus treated were maintained for four hours on an illuminated white background. The striking results obtained by Parker were confirmed, i.e., the distal pigment of the covered eye moved into the dark-adapted position while that of the exposed eye remained light adapted. But subsequent experiments showed that Canada balsam alone had an effect on the distal pigment. A minute drop of Canada balsam, without lampblack, was applied to an eye and the animal thus treated was kept under observation. In less than half an hour the distal pigment cells of the ommatidia, directly beneath the Canada balsam, had migrated towards the periphery of the eye, indicating that the positional changes observed with this method were not due to opacity of the lampblack; probably the xylene penetrated the cornea and brought about the changes in position of the distal pigment.

(*a*) *Clay method.* A series of experiments was carried out using modelling clay as an opaque covering for the eye. The position of each pigment was recorded in each experiment and is given below and in Table II(a).

TABLE II

The effect of covering one eye and exposing the other to direct, incident and reflected illumination. In animals of the (*a*) *series the eyes were covered with modelling clay after they had been kept on white backgrounds for three hours, and they were then placed on illuminated white and grey backgrounds. Animals of the* (*b*) *series were kept for three hours in darkness before their eyes were covered with modelling clay. Animals of series* (*c*) *were kept in a light-proof box as described in the text. The standard deviation has been calculated by the formula* $S.D. = \sqrt{\frac{\Sigma D^2}{n}}$.

	Numbers of animals	Conditions before experiment	Background	Time		Position of proximal pigment	Distal pigment index*
(a)	4	illumination	white	4 hours	exposed eye	light-adapted	.44±.048
					covered eye	dark-adapted	.44±.069
	6	illumination	grey	3 hours	exposed eye	light-adapted	.48±.060
					covered eye	dark-adapted	.51±.045
(b)	5	darkness	white	3 hours	exposed eye	light-adapted	.51±.027
					covered eye	dark-adapted	.60±.031
	5	darkness	black	3 hours	exposed eye	light-adapted	.59±.055
					covered eye	dark-adapted	.65±.060
(c)	3	illumination	—	2–3 hours	exposed eye	light-adapted	.49±.087
			—		covered eye	dark-adapted	.69±.098
	5	darkness	—	2–3 hours	exposed eye	light-adpated	.62±.071
			—		covered eye	dark-adapted	.69±.068

* An increase in the distal pigment index shows a movement towards the dark-adapted position.

(1) No changes were detected in the reflecting pigment.

(2) In all cases the proximal pigment of an exposed eye migrated distally in the retinular cells until it reached their distal extremities. On the other hand, the proximal pigment of the covered eye did not extend beyond the level of the tapetal layer, i.e., it occupied about half of the retinular cells above the basement membrane in each case. This is the position of the proximal pigment in animals maintained in darkness during the day. There was, therefore, a clear difference in the position of the proximal pigment in the exposed and the covered eye of each individual (Figs. 8 and 9).

(3) The positions of the distal pigments were observed under three different conditions of the animals. The first of these were animals which had been kept on illuminated white backgrounds before one of the eyes was covered, after which the animals were returned to illuminated white backgrounds. The distal pigments in these experiments showed no difference between the covered and the exposed eyes. In the second of these conditions the animals had previously been kept in darkness and one eye had been covered in very dim light. These animals were then kept on an

illuminated white background. In this case some slight difference between the covered and the exposed eyes was detected. Under the third condition the animals, previously kept in darkness, had one eye covered and were then placed on an illuminated black background. Here the difference in the position of the distal pigment in the exposed eye and the covered eye was significant. In many cases the distal retinal pigment index does not adequately represent this phenomenon, for the difference is a slight one, not easily expressed numerically, but clearly visible, especially in those animals in which the position of the distal pigment of both eyes approximated that found in darkness.

(*b*) *Box method.* The modelling clay method of covering the eye has two disadvantages: the body is exposed to illumination and light may enter the covered eye through the transparent eye-stalk. Verrier (1941) has reported that in *Leander squilla* covering of both eyes does not prevent light adaption of the distal retinal pigment if the body is exposed to illumination (her plates show that the proximal pigment of the covered eyes become partially dark-adapted like that of *Leander serratus* placed in darkness during the day). It is not surprising, therefore, that in the covered eye experiments described above the distal pigments generally became partially light-adapted.

In the box experiments, however, one eye only is exposed to illumination, while the rest of the body is in darkness. The results of these experiments are given in Table II(c). They differ from those in the previous experiments in that the distal pigment of the covered eye generally showed little or no light-adaption. The distal pigment of the illuminated eye showed a slight proximal migration which was always greater than that observed in the covered eye. (See Figs. 4, 5, and 6.)

The effect of sinus-gland removal

The effect of sinus-gland removal was studied in *Leander serratus* at Plymouth and in *Leander adspersus* at Naples. In some cases both sinus-glands were removed from an individual; in other cases the sinus-gland was removed from one eye-stalk and the other eye-stalk was ablated. The results obtained were substantially the same whichever method of sinus-gland removal was adopted. The animals were kept for ten days after the operation and then placed on a white background for 3–4 hours under powerful illumination. At the end of the experiment the animals were killed and the eyes were sectioned. A few animals without sinus-glands were kept in darkness as controls.

Migration of the proximal pigment was unaffected by the removal of the sinus-gland and invariably became light-adapted under illumination and dark-adapted in darkness. The extent of migration of this pigment was similar to that observed in normal individuals kept under similar conditions of illumination.

The position of the distal pigment in the eyes of the operated animals showed some variation in different individuals. In some individuals it attained the position of complete dark-adaptation (Fig. 4), even though the animals had been exposed to direct and powerful illumination; in some other individuals it underwent a slight proximal migration.

A few individuals of *Leander adsperus* were operated on for the removal of the sinus glands, and later, after a few days, one eye was covered with modelling clay. The results obtained by this means were similar to those obtained in *Leander serratus* by the box method, although the migration of the distal pigment in the illuminated eye was greater than that observed in *Leander serratus.*

DISCUSSION

The experiments which have been described in the present paper leave little room for doubt that the presence or absence of illumination has a direct effect on the cells containing pigment in the retina of *Leander serratus.* The migrations of the proximal pigment, and to a lesser degree those of the distal pigment, are greater in an exposed eye than in a covered one. It is necessary, therefore to re-examine the evidence for a humoral control of retinal pigment migration.

Evidence for the humoral control of proximal pigment migration depends on the injection experiments of Welsh (1939). An injection of eye-stalk extract caused the proximal pigment of Cambarus to migrate in darkness. It is clear from the experiments described above that the presence of the sinus-gland is not necessary for a migration of the proximal pigment in *Leander serratus* and *Leander adspersus.* Kleinholz (1948) has observed a similar persistance of proximal pigment migration after sinus-gland removal in Cambarus, and he has subsequently shown (1949b) that migrations of the proximal pigment, in response to illumination, continue in the isolated eye of Astacus in which the circulatory system no longer functions and the influence of the brain can no longer operate. The results obtained in the present experiments, by covering one eye, show that the proximal pigment responds directly to the presence or absence of illumination.

The evidence obtained from the study of persisting diurnal rhythms in *Leander serratus,* and in other species, shows that migration of the proximal pigment can take place in the absence of illumination. It is clear, therefore, that the control of the proximal pigment is a complex one, in which direct illumination is not the only factor.

The effect of injection experiments and sinus-gland removal show that the control of the migration of the distal retinal pigment is predominantly humoral. It is clear also, from the immobility of the distal pigment in certain individuals following sinus-gland removal, that the distal pigment cannot respond directly to illumination by migrating proximally. There remains, therefore, the puzzling dissimilarity between the exposed and covered eyes of single individuals in respect to their distal pigments (Fig. 4.). The present evidence suggests that light does have a direct effect on the distal pigment, but *only when the sinus-gland hormone is circulating in the blood stream.* If this is indeed the case, it is possible that light is in some way sensitizing the distal pigment cells to the hormone by which they are activated. Such a variation in the response of an effector to its activator is unusual and merits further investigation.

SUMMARY

1. The distal and the proximal retinal pigments of *Leander serratus* can alter their positions in response to light and darkness.

2. The reflecting pigment shows a great movement at night which persists as a diurnal rhythm in continued darkness, but during the day it is unaffected by change of illumination.

3. The extent of distal pigment migration depends on the shade of the background on which the animal is placed, if the intensity of incident illumination is constant.

4. In constant darkness the proximal pigment continues to migrate diurnally for some days, at least. At midnight it shows a greater degree of dark-adaption than it does during the day; the distal pigment cells do not exhibit any detectable persisting diurnal rhythm in continued darkness.

5. After removal of the sinus-gland the distal pigment, in most of the operated animals, attained the position of maximal dark-adaption and was unaffected by change of illumination; it therefore appears not to respond directly to illumination. On the other hand, the proximal pigment in animals without sinus glands exhibited the same responses to change of illumination as those observed in normal animals.

6. In a series of experiments one eye of an animal was covered and the other eye was illuminated. In all the experimental animals so treated the proximal pigment of the exposed eye became light-adapted, but that of the covered eye attained the position of dark-adaptation observed in animals kept in darkness during the day.

7. A slight difference between the position of the distal pigment in the exposed eye and the covered eye of an individual could sometimes be observed; the difference was most striking in those animals which were on a black background, and in those animals which were placed in a light-proof box (in darkness) with one eye protruding through the box to be illuminated.

8. The distal retinal pigment of *Leander serratus* does not respond directly to illumination when the sinus gland is absent, yet in normal animals direct illumination enhances the light adaptation of the distal pigment; possibly illumination sensitizes the distal pigment cells to the hormone by which they are activated. The migration of the proximal pigment under illumination appears to be independent of the sinus gland. The influence of direct illumination on the proximal pigments appears to be great.

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STUDIES IN THE PHYSIOLOGY OF COMMENSALISM.
I. THE POLYNOID GENUS ARCTONOË

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Significant work on the role of specific chemical factors in controlling host-parasite or host-commensal relationships has been published by Thorpe and Jones (1937) and by Welsh (1930, 1931). The former authors have demonstrated the significance of olfactory conditioning in host-selection by parasitic insects. Welsh has shown that the normally positively phototactic sign of the mite *Unionicola* is reversed by a substance released from its host, the fresh-water clam *Anodonta*, and also that the sign of three species of *Unionicola* occurring in clam hosts of different genera can be reversed by substances from their hosts alone. To the writer's knowledge, however, no experimental work has been done on any of the legion of host-parasite or host-commensal associations to be found in the sea, nor has it been demonstrated whether or not in any of these a chemical bond exists between host and partner.

The members of the polynoid genus *Arctonoë* present an interesting commensal complex. These worms can readily be collected in Puget Sound both in the intertidal zone and by dredging; along with their hosts they are in many ways admirably suited to experimental work. In Table I the host genera of the three described species of *Arctonoë* are listed, as given by Pettibone (1947) and the Berkeleys (1948). It is to be noted that *A. fragilis* (Baird) is listed as commensal with certain starfish only, while *A. pulchra* (Johnson) and *A. vittata* (Grube) are commensal with certain starfish, holothurians, gastropods, chitons and terebellids. The records would seem to show that some hosts (see arrows) may harbor two species of commensals, though there are no records at hand of an individual host with more than one species of commensal. The cucumber *Stichopus*, although not listed as a host of *vittata* by these authors, is included on the basis of commensals identified for the writer by Dr. Olga Hartman, according to whom the commensals of *Stichopus* may show some characteristics of *pulchra* and some of *vittata*.

While some commensal populations of *Arctonoë* appear to be relatively distinct on anatomical grounds (markings, condition of neuropodial setae and margins of elytra), others are variable and appear to include intergrades. By dealing with the complex as made up of populations on certain specific hosts it seemed that it might be possible to cast some light on the basic physiology of the partnerships and thereby, incidently, on the taxonomic problems involved. The primary question arose: "How are these worms bound to specific hosts, existing as they may in environments

TABLE I

Hosts of the Genus Arctonoë

	fragilis (Baird)	*pulchra* (Johnson)	*vittata* (Grube)
Asteroidea	Evasterias	Solaster ←	→ Solaster
	Luidia ←	→ Luidia	Dermasterias
	Stylasterias	Pteraster ←	→ Pteraster
	Orthasterias		Henricia
	Leptasterias		Crossaster
	Pisaster*		
Holothuroidea		Stichopus ←	→ (Stichopus)
Gastropoda		Megathura*	Diadora
			Puncturella
			Acmaea
			Cidarina
			Argobuccinium
Amphineura		Cryptochiton* ←	→ Cryptochiton
Polychaeta		Loimia*	Neoamphitrite

* Not recorded as hosts in Puget Sound.

where other species related to their hosts but without commensals are in close association or in actual contact with them?"

To the end of determining whether some specific chemical bond exists between commensal and host, studies were conducted at the Oceanographic Laboratories of the University of Washington at Friday Harbor, under a grant from the American Academy of Arts and Sciences. The writer wishes to express his appreciation to the Academy and to thank the Director of the Laboratories, the members of the staff and in particular Dr. Robert Hungate of Washington State College for their generosity and kind assistance.

MATERIAL

Worms of the beautiful form *A. fragilis* (Baird), commensal on the locally abundant inter-tidal star *Evasterias troschelii* Stimpson, were selected as the first animals to be used in these experiments and proved a happy choice for a number of reasons. *Evasterias* can be found in large numbers at certain points in Puget Sound where strong tidal currents prevent the deposition of sand or mud or the growth of extensive beds of algae. Stars were collected on a point of Orcas Island on Wasp Passage and on the pilings of the ferry landing at the village of Orcas. Although *Evasterias* has a more local distribution than the ever-present *Pisaster ochraceus,* when concentrations of the animals are found they are often in the same zone and in close association with *Pisaster, Pycnopodia, Solaster, Henricia, Leptasterias, Dermasterias* and other genera. Approximately thirty per cent of the *Evasterias* appeared to have commensal *Arctonoë*. The original observation of these commensals on hosts in close association with other genera of stars which are not regularly their hosts initiated these experiments.

Large numbers of the mud-star, *Luidia foliolata* (Grube), were collected by dredging in West Sound on the south side of Orcas Island. A high percentage of these stars had commensal *Arctonoë* (*pulchra-fragilis*?). This partnership did not, however, prove as satisfactory for experimental work as *Evasterias* and its commensal, because of the tendency of the mud-stars to autotomize their arms in the live-boxes and die.

Stichopus californicus (Stimpson) with attendant *Arctonoë* (*pulchra-vittata*?) could be collected by dredging or with a long rake almost anywhere in the Sound where there was soft bottom.

Although the large numbers of the form *A. vittata* commensal with the keyhole limpet *Diadora* made this association readily available, the extreme inactivity of these worms was such as to make their use in the experimental apparatus impossible.

It was found that host *Evasterias* and *Stichopus* could be kept in live-boxes for weeks without any apparent effect on the health or activity of their commensals. The worms were found to be extremely hardy and easy to handle. The name *fragilis* is certainly a misnomer and quite clearly the result of the observations of a worker who noticed that the worms break into fractions when placed in formalin or alcohol. In the course of the experiments worms were pulled from the host with forceps, manipulated with camel's-hair brushes, placed in the test apparatus, removed and replaced on their hosts many times without any apparent effect on their health and activity other than the occasional loss of an elytron. Twenty *Evasterias* with commensals were collected at the first of the summer and kept in live-boxes for two months, and in the tests to be described with *A. fragilis,* their worms were used exclusively. The hosts were fed bivalves of various species and when a star was feeding, its commensal could often be observed lying in the fold between the arms of the star with its head directed toward the everted stomach of the star, apparently stealing a meal.

METHODS

The essentially simple unit diagrammed in Figure 1 was designed to make commensals choose between two streams of water. It consisted of a glass Y-tube with a seven-and-a-half-inch stem of three-quarter-inch pyrex and two four-inch arms of one-half-inch pyrex at approximately right angles to each other. These arms were extended and bent inward by joining to them three-inch lengths of half-inch pyrex by means of rubber sleeves. This was done in order to eliminate turbulence and to give a smooth flow at the point-of-choice (B). At the open base of the Y-tube (A) was a stop-cock set in a rubber stopper, which could be removed from the Y to allow introduction of animals.

Two identical wooden aquaria with an internal dimension of 10″ × 10″ × 10″ were each connected to both arms of the Y, making it possible for alternate arms of the Y to be used as test arms. Vents at the bottoms of the aquaria were connected to the extended arms of the Y by $\frac{3}{16}$″ rubber tubing. By closing a pinch clamp on any one of the four rubber connections, flow in that part of the system could be completely stopped.

Commensals to be tested were separated from their hosts for twelve hours or more prior to an experiment and kept singly in dishes with circulating sea-water, on the theory that by such a procedure the threshold of the worms to "host-factor" might be lowered. Echinoderm hosts were placed in non-circulating, though aerated, sea-water in a glass aquarium and this, in turn, was kept partially immersed in a larger aquarium of circulating sea-water to maintain a low temperature (approxi-

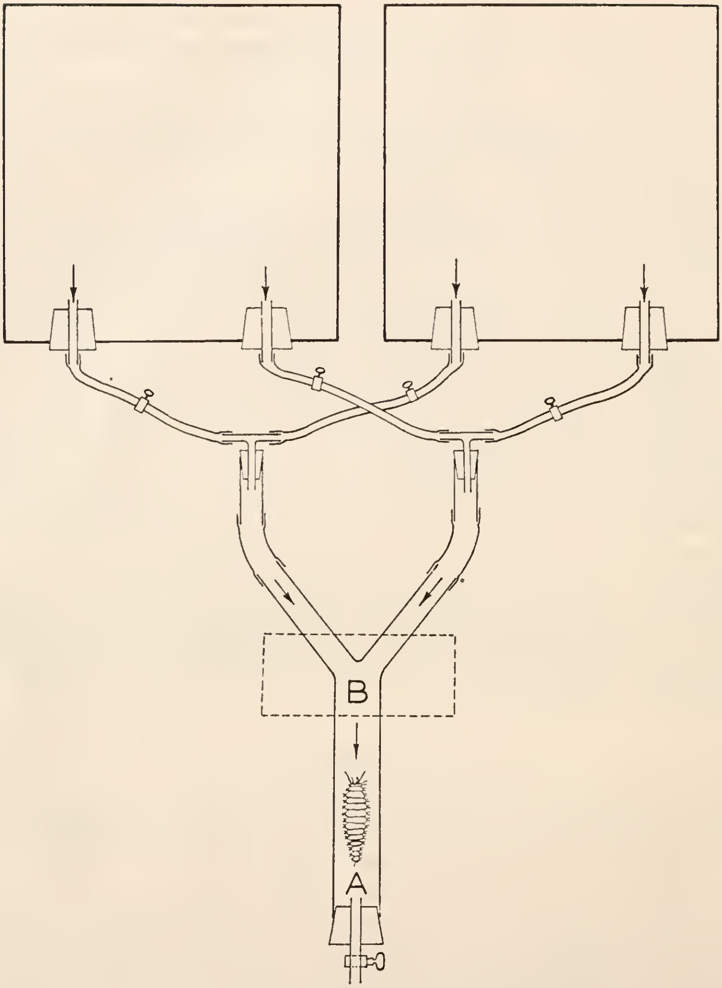

Figure 1. Plan of experimental apparatus.

mately 13.5° C.) over night. This was done in the hope that "host-factor" might accumulate in the water to be tested. An identical glass aquarium containing sea-water alone (or non-host to be tested against host) was treated in a similar manner. Immediately prior to a series of experiments the entire contents of the two glass aquaria including the echinoderms were transferred to the two wooden aquaria of the test apparatus. Each pinch clamp was opened to remove air from the system, both arms of the Y were flushed out from the sea-water or non-host aquarium, and the apparatus was ready for a run.

When a run was to be made, the stopper at A was removed with the four pinch clamps closed, and a worm introduced. The stopper was then replaced and the contained stop-cock opened to allow a slow but constant flow. Two of the pinch clamps were then opened at will to allow water from each aquarium to meet at the point-of-choice (B). When the worm had moved up to the point-of-choice and entered one of the arms of the Y, it was removed from the system, the choice recorded, and the apparatus readied for another run.

Every possible precaution was taken to prevent at the point-of-choice any other imbalance of physical or chemical factors than that desired. The level of water in the two aquaria was kept identical at the beginning of every individual run so that during the run the pressure in the two arms of the Y at the point-of-choice was by necessity equal, the heads above them being equal. The temperature was kept the same in both aquaria (13.5–15° C.).

Before any experiments were made flow tests were conducted using colored indicators. Figure 2 shows the head of a worm at the point-of-choice (Fig. 1, B) with the flow characteristics superimposed upon it. If an indicator was put into one aquarium, it was clearly demonstrated that the flow characteristics were such that if a chemical were to be present in one aquarium, the head of the worm and its tentacles would be asymmetrically stimulated. The two streams did not mix completely at the point-of-choice, there being a region of high concentration of indicator at one side, a narrow cone-shaped zone of mixing in the middle and a clear region without indicator at the opposite side. Farther down the stem and at the point of introduction (A) mixing was complete.

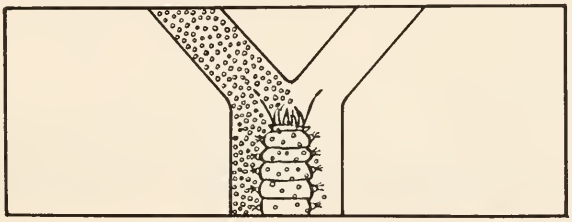

Figure 2. Enlargement of Figure 1, B. Flow characteristics as indicated by borax carmine, showing asymmetrical stimulation of tentacles of commensal at point-of-choice.

In addition, although worms were not strongly or negatively phototactic, in order to control differences in light as well as small differences in temperature, pressure, rate of flow or aeration, in any series of runs water from each aquarium was allowed to flow into alternate arms of the Y. For instance, if a series of runs were being made with a host in one aquarium and sea-water alone in the other, in the first run water from the host flowed into the left arm of the Y, in the next into the right arm, in the next into the left arm again, and so on alternately throughout the series. Between each and every individual run both arms were flushed out from the sea-water (or non-host) aquarium and between any series of tests the whole apparatus was thoroughly washed out with fresh sea-water. Both the glass and wooden aquaria were scrubbed and leached in fresh running sea-water for at least twelve hours. The wooden aquaria used to house hosts during runs were also periodically alternated.

Observations

In the data below the total number of times worms were placed in the test apparatus is recorded as the *"number of trials."*

If a worm moved up the stem of the Y and into either arm this is recorded as a *"run."*

If a worm failed within ten minutes from the time it was placed in the apparatus to reach the point-of-choice or enter either arm of the Y or if it reversed its position, this is recorded as a *"failure."*

The *"negative trials"* appearing in some of the tables below equal the sum of *"failures"* plus *"runs into the sea-water (or non-host) arm."*

Experiment No. 1. Are *Arctonoë fragilis* attracted by sea-water coming from an aquarium containing their host *Evasterias troschelii?*

No. of trials	43
No. of runs	35
No. of runs into *Evasterias* arm	33
No. of runs into sea-water arm	2
% runs into *Evasterias* arm	94.5%
Chi-square of choice (vs. 50/50)	27.4
P	$\ll .01$
No. of failures	8
% failures	18%
No. of worms used	13
No. of worms making five consecutive runs into *Evasterias* arm	5

It would appear from these data that the worms are attracted by some substance from the host aquarium and that they can distinguish between it and sea-water alone when they reach the point-of-choice.

Experiment No. 2. Is the ability of *A. fragilis* to determine which of the two arms of the Y contains sea-water from an *Evasterias* diminished if the other arm contains water from an aquarium containing a starfish of another genus, *Pisaster,* on which the worm is not commensal? Weight *Evasterias*—540 grams. Weight *Pisaster*—430 grams.

No. of trials	36
No. of runs	35
No. of runs into *Evasterias* arm	33
No. of runs into *Pisaster* arm	2
% runs into *Evasterias* arm	94.5%
Chi-square of choice	27.4
P	$\ll .01$
No. of failures	1
% failures	2%
No. of worms used	6
No. of worms making five consecutive runs into *Evasterias* arm	5

It would appear that the presence of the *Pisaster* in this experiment had no effect on the choice of the worms.

Experiment No. 3. What happens if in the above experiment we double the weight of non-host star in the other aquarium? Weight of host—489.5 grams. Weight of two *Pisaster*—866.1 grams.

No. of trials	37
No. of runs	36
No. of runs into *Evasterias* arm	31
No. of runs into *Pisaster* arm	5
% runs into *Evasterias* arm	86.1%
Chi-square of choice	18.68
P	$\ll .01$
No. of failures	1
% failures	2.7%
No. of worms used	6
No. of worms making five consecutive runs into *Evasterias* arm	4

It appears that doubling the weight of the non-host *Pisaster* had no significant effect on the ability of the worms to choose the arm of the Y in which water flowed from the host.

Combining all the above data for trials when the host was in the system:

No. of trials	116
No. of runs	106
No. of runs into *Evasterias* arm	97
No. of runs into sea-water or non-host arm	9
% runs into *Evasterias* arm	91.4%
Chi-square of choice	73.0
P	$\ll .01$
No. of failures	10
% failures	8.6%
No. of negative trials (9 plus 10)	19
No. of worms used	19
No. of worms making five consecutive runs into *Evasterias* arm	13

Experiment No. 4. How do *Arctonoë fragilis* collected from *Evasterias* behave when their own host is replaced in the test apparatus by a specimen of *Luidia foliolata* which is listed as an occasional host of *A. fragilis*? *Luidia*—weight approximately 920 grams.

No. of trials	35
No. of runs	14
No. of runs into *Luidia* arm	8
No. of runs into sea-water arm	6
% runs into *Luidia* arm	57%
Chi-square of choice	.285
P	.50–.95
No. of failures	21
% failures	72%
No. of negative trials (6 plus 21)	27
No. of worms used	9

It can be seen from the above that the distribution of runs into the *Luidia* arm and into the sea-water arm does not differ significantly from a chance distribution. Furthermore, if we compare the proportion of the runs into the *Luidia* arm to the negative trials (8/27) with the proportion, taken from the preceding sum of the data when the host was in the system, of the runs into the host arm to the negative trials (97/19), we see that the chi-square is 47.0 and the $P \ll .01$. It appears, therefore, that under these experimental conditions at least, *Luidia foliolata* has no attraction for the *A. fragilis* which are commensals of *Evasterias,* in spite of the fact that this worm has been described as occurring on *Luidia*.

Experiment No. 5. Are *Arctonoë* (*pulchra-fragilis?*) attracted by water coming from an aquarium containing their host *Luidia foliolata?* *Luidia*—weight approx. 920 grams.

No. of trials	35
No. of runs	20
No. of runs into *Luidia* arm	8
No. of runs into sea-water arm	12
% runs into *Luidia* arm	40%
Chi-square of choice	.80
P	.30–.50
No. of failures	15
No. of negative trials (15 plus 12)	27
No. of worms used	8

It appears that the worms cannot discern the presence of their host since the data for choice does not significantly differ from a chance distribution. That the behavior of the worms under these conditions is significantly different from that of worms in which there is evidence of a strong attraction can be seen if we compare the proportions of the number of runs into the host arm to the number of negative trials (8/27) with the same proportion for the sum of the data for *A. fragilis* and *Evasterias troschelii* (97/19). The chi-square is 47.0 and the P $\ll .01$.

Experiment No. 6. Can the above worms commensal with *Luidia* discern the presence in the system of the star *Evasterias* which has been shown to have a positive attraction for its own commensal *Arctonoë?*

No. of trials	18
No. of runs	5
No. of runs into *Evasterias* arm	2
No. of runs into sea-water arm	3
No. of failures	13
% failures	72%
No. of negative trials (13 plus 3)	16
No. of worms used	8

The very high proportion of negative trials to total trials (16/18) makes it clear that the substitution of an *Evasterias* for the host *Luidia* in the system has no effect on the behavior of these worms.

Experiment No. 7. Are *Arctonoë* (*pulchra-vittata?*) attracted by water coming from an aquarium containing their host *Stichopus californicus?* Host weight approx. 1000 gms.

No. of trials	56
No. of runs	50
No. of runs into *Stichopus* arm	45
No. of runs into sea-water arm	5
% runs into *Stichopus* arm	90%
Chi-square of choice	32.0
P	$\ll .01$
No. of failures	6
% failures	10.7%
No. of negative trials (6 plus 5)	11
No. of worms used	11

It would appear that these worms can clearly discern the presence of their host and distinguish between host-water and sea-water alone.

Experiment No. 8. Are *Arctonoë* commensal with *Stichopus* attracted by water from an aquarium containing another Holothurian, *Cucumaria miniata* (Brandt), found in the same general environment as their host? Combined weight of twelve *Cucumaria* in test aquarium—428 gms.

No. of trials	35
No. of runs	24
No. of runs into *Cucumaria* arm	9
No. of runs into sea-water arm	15
% runs into *Cucumaria* arm	37.5%
Chi-square of choice	1.5
P	.20–.30
No. of failures	11
% failures	31.5%
No. of negative trials (11 plus 15)	26
No. of worms used	6

It is clear that in this series of runs the distribution beyond the point-of-choice did not significantly differ from a chance one. That the behavior of the worms differed significantly from their behavior when the host was in the system can be seen when we compare the proportion of runs into the *Cucumaria* arm to negative trials (9/26) with the similar proportion from the preceding table when the host was in the system (45/11). The chi-square is 26.2 and the $P \ll .01$.

Experiment No. 9. What happens if, in the test apparatus, we cross hosts of two partnerships in which a clear attraction has been demonstrated? How do worms commensal with *Stichopus* behave if a specimen of *Evasterias troschelii* replaces their host in the system?

Series A. *Evasterias* weight approx. 425 grams

No. of trials	15
No. of runs	13
No. of runs into *Evasterias* arm	5
No. of runs into sea-water arm	8
Chi-square of choice	.68
P	.30–.50
No. of failures	2
No. of worms used	5

Series B. *Evasterias* weight approx. 950 grams

No. of trials	25
No. of runs	15
No. of runs into *Evasterias* arm	11
No. of runs into sea-water arm	4
Chi-square of choice	3.2
P	.05–.10
No. of failures	10
No. of worms used	5

It can be seen that in both series A and B the distribution beyond the point-of-choice did not significantly differ from a chance one. Comparing the proportions of the number of runs into each arm in both sets of data we see that in series B in which the weight of *Evasterias* was increased in order to equal more nearly that of the host *Stichopus,* the runs into the *Evasterias* arm were in the majority. However, the number of failures (10) was high for an experiment demonstrating an attraction. A longer series of trials would have to be made in order to determine whether commensals from *Stichopus* may be significantly attracted by water from a large weight of their relative's host, *Evasterias.*

In order to compare the behavior of the worms in these two series of experiments with their behavior when the host was in the system, we may combine the data from series A and B.

No. of trials	40
No. of runs	28
No. of runs into *Evasterias* arm	16
No. of runs into sea-water arm	12
No. of failures	12
No. of negative trials (12 plus 12)	24
No. of worms used	10

That the behavior of the worms differed from their behavior when the host was in the system can be seen when we compare the proportion of runs into the *Evasterias* arm to negative trials (16/24) with the similar proportion when the host was in the system (45/11). Here the chi-square is 15.7 and the $P \ll .01$.

Experiment No. 10. How do worms commensal with *Evasterias* behave if a specimen of *Stichopus californicus* replaces their host in the system?:

No. of trials	35
No. of runs	33
No. of runs into *Stichopus* arm	17
No. of runs into sea-water arm	16
No. of failures	2
% failures	5.6%
No. of negative trials (2 plus 16)	18
No. of worms used	7

It is clear that in this experiment the data of choice did not differ from a chance distribution. That the behavior of the worms differed from their behavior when their host was in the system can be seen when we compare the proportion of runs into the *Stichopus* arm to negative trials (17/18) with the similar proportion from the sum of the data for experiments no. 1, 2 and 3 (97/19). Here the chi-square is 17.6 and the $P \ll .01$.

Discussion

The data above present clear statistical evidence that certain echinoderm hosts produce a specific diffusable substance that acts as a powerful attractant for their polychaete commensals. However, the data cannot describe the characteristic behavior of worms when they are under the influence of the attractant. Tested against sea-water alone, *A. fragilis* showed a random distribution of choice, but 8 of 21 trials were failures, and in the majority of the remainder of the runs the worms were sluggish, took the full ten minutes to reach the point-of-choice, and showed a decided lack of interest in moving up the stem. This behavior was more or less characteristic of all tests when hosts were not in the system and of tests with the mud-star, *Luidia foliolata*. Tested against hosts, however, the characteristic behavior of worms was to hesitate for a brief period at the point of entrance and then suddenly to become activated and move up the stem, waving the head from side to side in searching movements. The delay at the point of entrance appeared in these experiments to be about the same time it took indicator to reach this point in the preliminary flow-tests.

In a number of experiments in which positive results were obtained it is clear that some of the runs into the sea-water or non-host arm were the result of the worm moving so swiftly up the stem of the Y under the influence of "host-factor" that without stopping at the point-of-choice to sample the streams, it simply moved into that arm of the Y nearest the side of the stem to which it was appressed when it reached the point-of-choice. A number of times during the course of the experiments worms were observed to move part way into the sea-water or non-host arm, then pull back and enter the host arm.

The failure of *Arctonoë* commensal with *Luidia* to respond to their own hosts is unexplained. However, it was impossible to obtain an entirely uninjured host for use in the test apparatus and if a whole one was put into the apparatus it soon autotomized one or more arms. Perhaps the failure to obtain a response by commensals depended upon the release of injury substances from these stars.

It would appear that other echinoderms not distantly related to hosts, but without commensals, have no attraction for the commensals. However, a survey of many more forms should be made before this can be certain.

Reciprocal experiments indicate that "host-factor" produced by the host of one partnership has no attraction for commensals of another partnership, in spite of the fact that the commensals may be extremely closely related. However, little light has been cast by these preliminary experiments on the interesting phenomenon of the restriction of what appear to be single commensal species to certain species only of a number of totally unrelated classes of hosts (see Table I). By similar tests it should be possible to give evidence whether or not attractants of two unrelated hosts of the same commensal species may be similar.

It is clearly apparent that details of the life-histories of the forms of the genus *Arctonoë* are needed, involving examination by specialists of extensive series of different age groups of commensals from each of the many host species. Although in the differentiation of the three forms it is almost certain that genetic factors are involved, it would be of great interest to determine whether or not the early stages of one form could be induced to develop on the host of another form and if so, whether morphological as well as physiological adaptations to the new host might occur independent of genetic factors. In certain species of insect parasites, structural polymorphism dependent upon host has been clearly demonstrated by Salt (1937) and others.

That organisms by undergoing their development in contact with certain specific chemical factors may be physiologically conditioned to respond positively to them has been demonstrated by Thorpe (1939) in his work with Drosophila. The work cast some light on earlier observations by Thorpe and Jones (1937) that the wasp *Nemeritis* (Hymenoptera: Ichneumonidae), normally a parasite of the Wax-moth *Ephestia,* could be conditioned to give a positive olfactory response to the Wax-moth *Meliphora* if it had undergone its development on this abnormal host. Similar experiments could perhaps be conducted using marine partnerships; they might be initiated by using such forms as the polynoid *Halosydna brevisetosa* Kinberg, in which both free-living individuals and morphologically very different individuals commensal with certain terebellids are readily available. It is quite possible that just as in certain terrestrial host-parasite relationships (Thorpe and Jones, 1937) we have in these intimate marine associations the factor of olfactory conditioning acting as a mechanism of isolation, which Cushing (1941) suggests may be "a physiological factor acting upon population mechanics that does not depend upon genetic changes for changes in its specificity of action."

The general occurrence of chemical factors as controlling agents of host-commensal relationships in the sea must be surveyed by adapting our apparatus so that members of other phyla can be used, and finally an investigation must be made of the chemical nature of attractants.

SUMMARY

1. Apparatus was developed which made it possible to determine whether commensals of the polynoid genus *Arctonoë* are attracted by specific substances to their hosts and whether they can distinguish between water coming from an aquarium containing their host and sea-water alone or water from non-host.

2. *Arctonoë fragilis* (Baird) commensal with the star *Evasterias troschelii* Stimpson are clearly attracted to their host and can distinguish between water coming from their host and sea-water alone. *Arctonoë* (*pulchra-vittata*?) commensal with the cucumber *Stichopus* are likewise attracted to their host.

3. *Arctonoë fragilis* commensal with *Evasterias* are not attracted to the non-host star, *Pisaster,* occurring in the same immediate environment as their host. *Arctonoë* (*pulchra-vittata*?) commensal with the cucumber *Stichopus* are likewise not attracted to the non-host *Cucumaria.*

4. *Arctonoë* (*pulchra-fragilis*?) commensal with the mud-star *Luidia* are not attracted, under these experimental conditions at least, to their own host. *Arctonoë fragilis* commensal with *Evasterias* are not attracted to the mud-star *Luidia,* in spite of the fact that *Luidia* may be a host of *fragilis.* These results are perhaps inconclusive, because injury substances may have been released into the apparatus by the mud-stars.

5. Cross experiments (*A. fragilis* from *Evasterias* tested against *Stichopus* and *A. pulchra-vittata* from *Stichopus* tested against *Evasterias*) indicate that attractants released by the two hosts are not the same, since neither commensal is attracted to its relative's host.

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THE EFFECTS OF SULFANILAMIDE UPON THE OXYGEN UPTAKE OF MITOTICALLY ACTIVE AND BLOCKED GRASSHOPPER EMBRYOS [1]

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The effects of sulfonamides upon bacteria have been repeatedly pointed out and various ideas as to their fundamental action in such cases have been advanced (Fildes, 1940; Woods and Fildes, 1940; Woods, 1940; Eyster, 1943; Steers and Sevag, 1949). That sulfonamides markedly interfere with such general cell activities as division and growth seems also well established (Fisher, Henry and Low, 1944). More specific effects of these reagents upon cellular enzyme systems have also been investigated and rather striking concepts of their modes of action demonstrated (Johnson and Chase, 1942; Mann and Keilin, 1940; Johnson, Eyring and Williams, 1942). Marked inhibitions of cell division, oxygen consumption and enzyme activity have been reported and of especial interest are the relations found between the effects of sulfanilamide upon oxygen uptake and cell division in various types of animal and plant cells (Fisher, Henry and Low, 1944). Fisher, Henry and Low (1944), for the sea urchin egg, have shown that when the cell division is stopped by sulfanilamide, the oxygen uptake and especially the activity system is also completely inhibited. In this respect the action of sulfanilamide seems to resemble that of certain narcotics (Johnson, 1942).

Since the embryo of the grasshopper in its development at constant temperature (25° C.) normally goes through a block or diapause period in which mitotic activity is completely stopped it becomes of some interest to determine the action of sulfanilamide on it during both the blocked and active phases of its development. Morphologically similar embryos in mitotically active and blocked states are readily available and lend themselves to such an investigation (Bodine and Boell, 1936). The present paper, therefore, deals with data concerning the effect of sulfanilamide upon the oxygen uptake of morphologically identical grasshopper embryos both in mitotically active and blocked conditions.

MATERIAL AND METHODS

Embryos, free from yolk, were obtained from eggs of the grasshopper, *Melanoplus differentialis*, as previously noted (Bodine and Boell, 1936). Oxygen determinations were carried out at 25° C. with Warburg manometers, using respiration flasks of 5 ml. capacity. Embryos were suspended in phosphate buffered (pH 6.8) NaCl solution (0.65 per cent) and solutions of sulfanilamide were also made up

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in it. Further details of procedure have already been described (Bodine and Fitzgerald, 1949).

RESULTS

The effects of sulfanilamide, ranging in concentration from 0.005 M. to 0.06 M., on the oxygen uptake of both active and blocked embryos were determined. Results of many such experiments are graphically shown in Figure 1. An inspection of this figure shows that sulfanilamide depresses the rate of oxygen uptake of both active and blocked cells to approximately 50 per cent of the normal value. No significant differences in responses between the mitotically active and blocked cells have been found (Table I). The effects of sulfanilamide upon the oxygen uptake do not seem to be conditioned by the mitotic activity of the cells of the embryos. By choosing embryos in various degrees of diapause or cellular block (as shown by rate of oxygen uptake) it is possible to show that the relative effects of sulfanilamide are similar in all cases. In Figure 2 are graphically shown the results of several experiments using 0.06 M. sulfanilamide and embryos in different levels of block. The degree of block or diapause in these embryos is indicated by their rates of oxygen uptake, i.e., embryos in complete block have much lower rates of oxygen consumption than similar ones out of block and actively developing (Bodine and

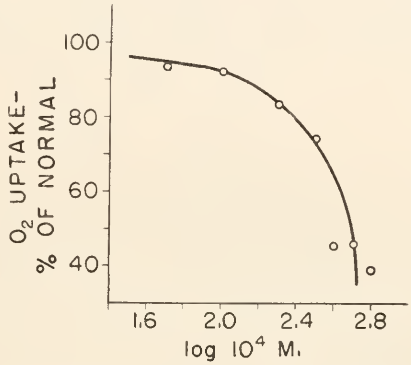

FIGURE 1. Shows the effect of sulfanilamide on the oxygen uptake of active and blocked embryos. Abscissa, log 10^4 M. sulfanilamide; ordinate, relative oxygen uptake rate, taking O_2 uptake of non-treated embryos as 100%. Circles represent averages of all experiments.

TABLE I

Inhibition of O_2 uptake in % of normal for embryos

Diapause	Postdiapause
44	70
48	55
69	58
68	74
65	43
49	
48	Av. = 60.0
56	
Av. = 57.2	

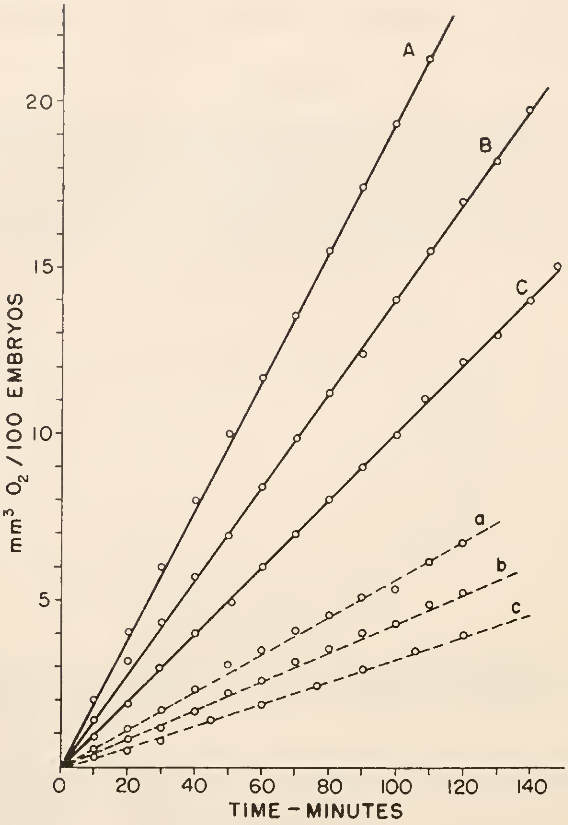

FIGURE 2. Shows effect of 0.06 M. sulfanilamide on the oxygen uptake of morphologically similar diapause and postdiapause embryos. Abscissa, time in minutes; Ordinate, mm.^{3} O_2/100 embryos. A = postdiapause embryos (2 days at 25° C.) in Ringer. a = same embryos exposed to sulfanilamide. B = embryos coming out of diapause. b = same exposed to drug. C = embryos in complete block or diapause. c = same exposed to drug.

Boell, 1936). An inspection of Figure 2 shows that for two-hour exposures to sulfanilamide the relative effects of the drug on the oxygen uptake of these embryos are practically constant throughout. It seems, therefore, that for the grasshopper embryo the particular states of their cellular activity have little to do with their response to sulfanilamide especially as regards their oxygen uptake. It is of some interest to note that the relative amounts of sulfanilamide sensitive (approx. 60–70 per cent) and sulfanilamide insensitive (approx. 30–40 per cent) fractions of the respiration seem to be practically identical in the mitotically active and blocked cells. The inhibitory effects of sulfanilamide for both types of embryos, except for prolonged exposures, are quite reversible.

DISCUSSION

The above results for the grasshopper embryo appear quite different from those found for the fertilized and unfertilized sea urchin egg (Fisher, Henry and Low, 1944). Fertilized sea urchin eggs behave quite like the grasshopper embryo while unfertilized eggs seem to be quite resistant to the action of sulfanilamide. The relative degrees of inhibition of the oxygen uptake for the fertilized sea urchin egg and the grasshopper embryo are quite similar (55–60 per cent). No cytological examinations of sulfanilamide treated active grasshopper embryos have been made to determine possible cellular effects and especially as regards effects on cell division. However, the blocked grasshopper embryo contains no mitotically active cells when in true diapause (Slifer, 1932) and its respiratory rate perhaps represents a real basal one. To what extent one is justified in comparing the physiological differences in response to sulfanilamide of the sea urchin egg and the embryonic cells of the grasshopper is quite an open question. For the grasshopper embryo one may reasonably conclude, however, that the inhibitory effects of sulfanilamide on the oxygen uptake seem to be quite independent of the mitotic behavior of the embryonic cells.

SUMMARY

1. The effects of sulfanilamide upon the oxygen uptake of mitotically active and blocked grasshopper embryos have been determined.

2. Sulfanilamide depresses the rate of oxygen consumption in both active and blocked embryos in a relatively similar fashion.

3. The sulfanilamide sensitive and insensitive fractions of the respiration are approximately constant for both active and blocked embryonic cells.

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DORSALIZATION OF THE VENTRAL MARGINAL ZONE OF THE TRITURUS GASTRULA. I. AMMONIA-TREATMENT OF THE MEDIO-VENTRAL MARGINAL ZONE[1]

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INTRODUCTION

In the early gastrula of amphibians the material for the notochord and mesoderm is localized between the animal and vegetative hemispheres in the form of a ring. This marginal zone is most extensive at its medio-dorsal section, which extends well above the equator, and represents the broadest section of the ring. As one proceeds from this part towards the opposite medio-ventral section, the ring becomes gradually narrower, its upper boundary gradually lower, and its narrowest and lowest limit, the medio-ventral section, lies opposite the future dorsal lip of the blastopore.

The distribution of the presumptive organ rudiments of the trunk mesoderm within this zone reflects the dorso-ventral sequence of the rudiments in the completed embryo. Medio-dorsally lies the presumptive notochord, on both sides of it the presumptive myotomes, which are followed by the presumptive pronephros and lateral plate, and finally the presumptive blood island, which occupies the medio-ventral section of the marginal zone.

This sequence of the prospective values within the marginal zone is again reflected in the mode of differentiation of the isolated sections of the marginal zone cultured in Holtfreter solution (Holtfreter, 1938). Although here the differentiation of the individual sections does not faithfully realize their prospective value, the dorso-ventral sequence of morphogenesis is in its general form clearly recognizable. The ventral section of the marginal zone differentiates blood island, nephric tubules and the derivatives of the lateral plate. However, neither notochord nor muscle is formed in the isolate from this section. In contrast to this the differentiation of the notochord and muscle is the predominant feature of the isolated dorsal section. It must be kept in mind, however, that especially in the dorsal section the "regulative" tendency is at work to develop more than the prospective value, so that a part of the explant from the dorsal section has always the chance to become one of the more ventral mesodermal rudiments or even ectodermal tissue.

If comparable sections of the trunk mesoderm are isolated later (at the beginning phase of neurulation) and cultured in epidermis vesicles, the individual sections can be distinguished more clearly through the mode of their differentiation (Yamada, 1939, 1940). But even in this case the prospective value is not always faithfully followed. Further, it is demonstrated that by adding a piece of the presumptive notochord to the individual sections of the mesoderm of the neurula it is possible to alter the mode of differentiation of each isolate. This effect of the notochord consists in shifting the mode of differentiation of the isolate toward the dorsal side. The presumptive blood island which differentiates blood island, mesothelium and blood vessels in isolation, gives rise to nephric tubules and only a small quantity of blood cells, if it is combined with notochord material. The presumptive pronephros, which develops, in isolation, nephric tubules and blood cells, shows clear differentiation of muscle cells in the direct vicinity of the implanted notochord tissue. Presumptive myotome, which in isolation gives rise to nephric tubules besides undifferentiated myoblasts, develops muscle cells with definite fibrils on implantation of the notochord material. Thus the effect of the notochord on the morphogenesis of the mesoderm can be designated as "dorsalization." From these results it may be expected that also the isolated ventral marginal zone of the gastrula could be dorsalized by appropriate methods, perhaps with more profound results, since the material must be more plastic in the gastrula than in the neurula.

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It is well known that the presumptive ectoderm of the early gastrula reacts to many chemical stimuli by undergoing neural differentiation. The most typical case may be the neuralization of the isolated gastrula ectoderm caused by a sublethal cytolysis, as demonstrated by Holtfreter (1945, 1947). Through brief treatment of the isolated gastrula ectoderm with a solution of extreme pH (higher than 9.4 or lower than 5.0), Ca-free Holtfreter solution, distilled water and 10 per cent alcohol, he obtained differentiation of brain, eye, placodes, mesenchyme and melanophores. Thus the cephalic ectodermal structures and mesectodermal components were induced, while the more caudal ectodermal structures, such as the otocyst and spinal cord, and the mesodermal structures were wholly absent. The arrangement and form of the induced rudiments were, however, highly atypical. The same seems to hold also for "self-induced" explants obtained by Barth (1942) and Holtfreter (1944) and for the explants of Shen (1942) cultured in a dilute solution of a hydrocarbon. Morphogenetic response of the isolated ectoderm to implantation of a protein belongs also to this category (Yamada, 1947). Here the induced structures are more diversified and typically organized than in the above cases.

All these inductions of the neural differentiation in the ectoderm are dorsalizations as defined above. This may become clear if one recalls the fact that epidermal differentiation is a ventral mode of differentiation, while brain differentiation is a dorsal one, according to the topographical relation which can be visualized, for instance, in a cross section of the anterior part of an early neurula. Between two extreme levels lie the intermediate levels of the sense organs, placodes and mesectoderm. Together they constitute the dorso-ventral scale of the anterior ectoderm. Induction or neuralization is then nothing but the conversion of the ventral mode into a more dorsal one. Thus the sublethal cytolysis dorsalizes the isolated ectoderm, which untreated tends to adopt the ventral mode of differentiation. Now in the double-potential theory proposed by the present author (Yamada, 1947, see below) the dorso-ventrality of the morphogenesis has a common basis in the ectoderm as well as in the mesoderm, and consequently the internal factor which regulates it in normal development may be of the same quality throughout the embryo. From this it may be expected that any factor which has a dorsalizing effect on the competent ectoderm can dorsalize the development of the mesoderm, if it is given in an appropriate condition. This theoretical expectation is tested in the following experiments.

As the dorsalizing procedure a brief exposure to a solution of ammonia was adopted. At first this treatment was tested on the isolated ectoderm of the gastrula of *Triturus pyrrhogaster* (control series I), and after its dorsalizing effect was ascertained, the isolated medio-ventral marginal zone of the gastrula of the same species was treated in the same way (experimental series). Parallel to this series, the untreated medio-ventral and latero-ventral marginal zones were isolated and cultured as the control of the reacting system (control series II).

Ammonia was used in the experiment since it actually is concentrated in the dorsal regions of the amphibian embryo during the phase of gastrulation and neurulation (Boell, Needham and Rogers, 1939), and may have the role of a mediating factor in morphogenetic induction.

Material and Technique

The eggs of *Triturus pyrrhogaster* were obtained through implantation of ox hypophysis in the mature female, and fertilized naturally or artificially. Operations and culture were done under sterile conditions. The egg capsule was washed with 60 per cent alcohol for 50 seconds, thoroughly rinsed in sterile Holtfreter solution and then removed with fine scissors sterilized with dry heat. After freeing from the vitelline membrane with forceps, the embryos were operated on with a glass needle on the Schotté ring (Yamada, 1937). Standard Holtfreter's solution (pH 7.2–7.6) was used exclusively for operation and culture. Sodium bicarbonate for the Holtfreter solution was sterilized with 60 per cent alcohol and after drying dissolved in sterile distilled water and then added to the unbuffered solution which had been boiled and cooled beforehand. The solution of ammonia used for the shock treatment was prepared immediately before use by adding 0.01 cc. of 20 per cent ammonia solution to 10 cc. of Holtfreter solution. Its pH was roughly estimated to be 11.8–12.2. The condition of the laboratory did not permit the regulation of culture temperatures which fluctuated between 12° and 26° C. However, no essential effect was noted other than a variation in the velocity of development. The isolates were cultured in covered glass dishes of 25 cc. volume. After culturing for seven to eighteen days the isolates were fixed in Bouin's fluid, stained in bulk with borax carmine, sectioned at 12 μ and stained with picro—"Blauschwarz."

The developmental stage of the operated embryo was classified according to the following criteria: Stage I: The youngest gastrula with the first sign of the blastopore as a pigmented patch; Stage II: Gastrula shortly after formation of groove of invagination; Stage III: The blastopore crescent-shaped.

Control Series I: Shock Treatment of the Isolated Gastrula Ectoderm with Ammonia

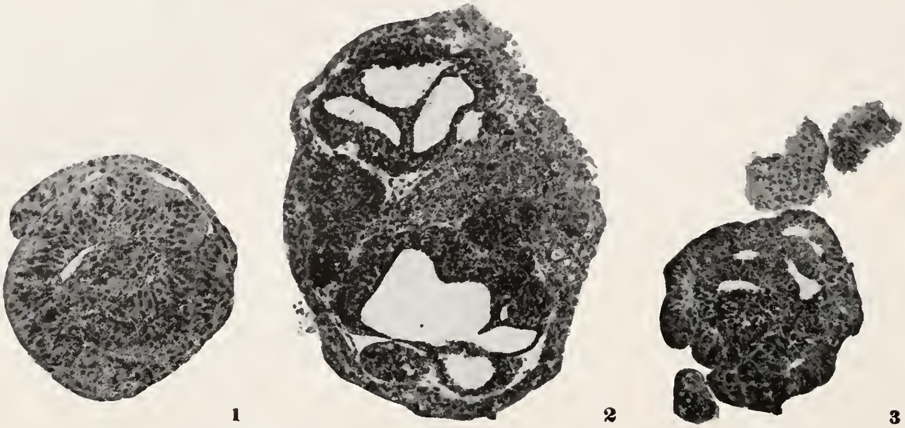
It was expected that the isolated ectoderm of the early gastrula of *Triturus pyrrhogaster* would differentiate cephalic ectodermal structures if exposed temporarily to a solution of ammonia, just like the isolated ectoderm of *Triturus torosus* (Holtfreter, 1945, 1947).

The presumptive ectoderm of the early gastrula was excised and put in the dilute solution of ammonia (see above). Special caution was taken not to include in the isolate any cells from the marginal zone. After a latent period of ca. 50 seconds the explant began to disintegrate. The first sign was disruption and break-down of the pigmented surface into small fragments. This process was often accompanied by inverse curling of the explant edge, which resulted in the formation of a cup with the original internal surface occupying the convex outside. This cup persisted only for a while. Then the individual cells rounded up, became transparent and swollen and burst out of the aggregate. As the disaggregation progressed further a sticky transparent colloid was released. Thus within three to four minutes the original contour of the explants was almost lost. The velocity of the disintegration varied considerably, perhaps owing to the differences in the state of the curling and size of the explants. Before the explants were completely disaggregated they were transferred to another dish containing Holtfreter solution. Here the cells became less and less transparent and a part of them adhered to each other to form a solid aggregate of varying size, while other cells remained free on the bottom of the dish and were later discarded. Five explants were treated simultaneously with ammonia solution, and the number of the resulting aggregates varied from two to three. An aggregation of cells from two different isolates to form a common aggregate was a usual occurrence. The solid aggregate was then further transferred to the second dish with Holtfreter solution and cultured as usual. The surface of the aggregate was almost perfectly free of pigment and seemed to be occupied by the originally internal surface of the ectoderm. In the course of the subsequent three days a considerable number of cells were discharged from the reaggregated explant. In most cases the latter became a more or less regular, compact spheroid. No extensive adhesion to the glass bottom was observed. The formation of irregular small folds which are characteristic for the untreated ectoderm explant of this species occurred only in four of forty explants examined. On the other hand, no neural plate could be observed on the surface of our aggregates. This deserves attention in so far as the isolated ectoderm of this species shows a very clear neural plate under the inductive influence of chemicals and the organizer. However, by dissecting the aggregates two to three days after the ammonia-treatment, small strongly pigmented areas were found in its interior, scattered among the pigment-free cells. These areas are derived perhaps from the original surface of the ectoderm and have the significance of a neural plate. Probably neural differentiation originates in connection with them.

Of forty microscopically examined explants, thirty-one (77 per cent) showed neural differentiation. Four of the nine without neural differentiation belonged to the afore-mentioned type with many irregular folds. In sections, they cannot be distinguished from the untreated explant of *Triturus* ectoderm, being an unorganized mass of epidermis cells. The five remaining explants without neural differentiation are spherical in shape and consist of tightly condensed epidermal cells. Within them are encountered many cysts which often enclose cytolyzed cell masses besides densely pigmented cells, suggesting severe disturbance of the metabolism.

The structures encountered in the neuralized explants may be classified as brain-type, eye-type, neural mass, nose-type, lentoid, atypical neural balls, mesenchyme, chromatophores and frontal glands. It must be added, however, that the classification is of a rather provisional character and does not mean necessarily the definitive identity of the induced structure with the corresponding normal rudiments (see below).

Brain-type. Of twenty-nine brain-like bodies three show a rather normal form (Fig. 1). The typical appearance is often emphasized by the presence of nasal placodes closely applied to the "brain." In the other brain-like bodies the morphology is atypical in different degrees. The most atypical state is attained when an enormous body is furnished with a number of highly irregular lumina. One of six such cases is shown in Figure 2.



FIGURES 1–3. Neural tissues induced in the isolated ectoderm through exposure to ammonia solution. Fig. 1. A brain-like structure with a "nasal placode" on the right side. Fig. 2. A large explant showing two extensive brain-like structures with many lumina, accompanied by some placodes and mesenchyme. Fig. 3. An irregular brain-like vesicle accompanied by epidermal balls with frontal glands. × ca. 60.

Eye-type. In only two cases eye-like tissue is developed in connection with the brain-type differentiation. One of them is provided with a pigment layer, while the other is accompanied by a lentoid.

Neural mass. A group of neuralized cells without definite morphological characterization. This occurs regularly when small explants are perfectly neuralized and no epidermis exists to cover the neural tissue. Seven such cases are recorded.

Neural balls. This category also has no corresponding normal structure. It is probably heterogeneous, comprising some neural vesicles which are too small to be called a brain, and some bodies of placodal nature often enclosing large pigment-vacuoles. Eleven neural balls are differentiated in eight explants.

Nose-type. Of eighteen nasal placodes obtained, thirteen are closely applied to the brain and show a fairly normal condition (Fig. 1). Two others are differentiated without accompanying neural tissue.

Mesenchyme and melanophores. These elements are differentiated definitely only in one explant in close relation to the induced neural tissue. They cannot be distinguished from the normal ones.

Frontal glands. Typically differentiated glands are found in nine explants. They often form irregular groups with epidermal cells and are attached to the neuralized cell-mass as a protuberance (Fig. 3).

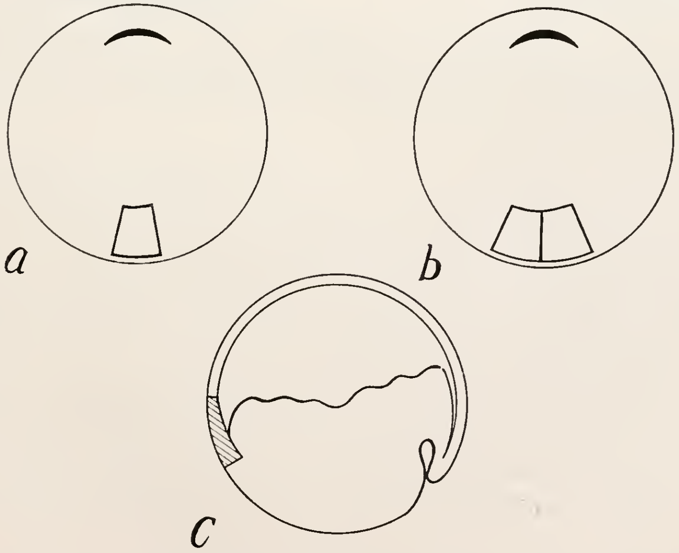
The size of the explants varied from 0.32 × 0.27 mm. to 1.28 × 1.05 mm. (expressed in the major and minor axes of the fixed explants, which are assumed to be an ellipsoid). Neuralization occurred in the smallest as well as in the largest ones. Often the smaller explants are perfectly neuralized, while in the larger ones the morphogenesis of the induced structures progressed better. In six cases all the cells of the aggregate are neuralized so that the neural tissues are exposed on all sides to the medium. But even in such a state some lumina developed inside the neural mass and endowed it with a brain-like appearance (Fig. 3).

Summarizing, we can say that through the ammonia-treatment of the gastrula ectoderm only cephalic ectodermal or mesectodermal differentiations are induced, while the mesodermal or relatively caudal ectodermal differentiations are wholly absent. The results conform well with the findings of Holtfreter (1944a, 1947).

It may be added that the same treatment was carried out on a number of isolated pieces of ectoderm from the late gastrula and early neurula. They were all underlain with endomesoderm. Most of them did not recover from the disintegration. Only two explants from the presumptive epidermis of the gastrula with large yolk-plug were successfully cultured. Upon examination in sections, one of them shows the brain-type and the other the nose-type differentiation.

CONTROL SERIES II: DIFFERENTIATION OF THE ISOLATED VENTRAL MARGINAL ZONE

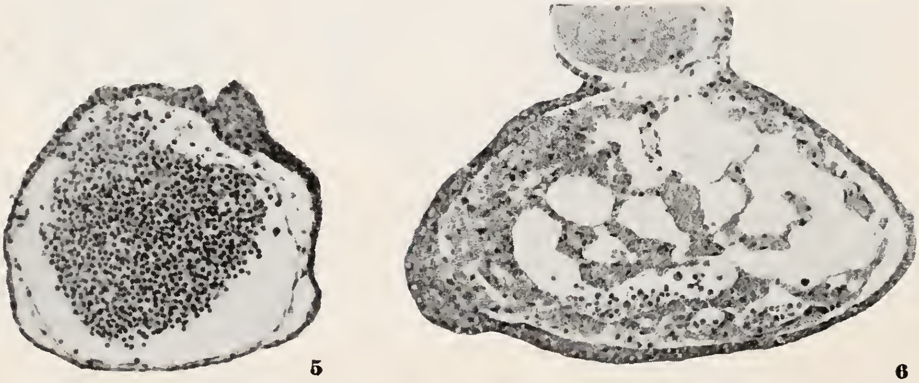
A part of the ventral marginal zone from the early gastrula was isolated and cultured in Holtfreter solution. Two different ways were adopted to obtain the isolate. In series IIa two symmetrical sectors of the marginal zone were extended ca. 20° from the ventral median line on both sides (latero-ventral section), while in series IIb the sector of 20° lying exactly medio-ventrally was isolated (Fig. 4a–c). Utmost caution was taken against inclusion of the cells from the adjacent



FIGURES 4a–c. Scheme of operation. The early gastrula, in *a* and *b* viewed from the vegetative pole, in *c* shown in the median section. Fig. 4a. Operation for the "medio-ventral" section of the marginal zone. Fig. 4b. Operation for the "latero-ventral" section of the marginal zone.

sectors of the marginal zone in the isolate. The isolated pieces contained however, besides the cells of the marginal zone, some cells from the adjoining ectoderm and endoderm. This inclusion was intentional because for a good histological differentiation of mesoderm cells the presence of cells from other germ layers, which later furnish a covering tissue, is a necessary condition. The isolated piece was in most cases fused with other pieces into a single explant and cultured. The number of pieces constituting one explant varied from one to six. Several examples will be described:

VRK 108. From five gastrulae of stage II the medio-ventral sectors of the marginal zone were isolated and all were fused together in an explant and cultured in Holtfreter solution. One day after the operation the endo-mesodermal part of the explant formed a large whitish hemisphere, on which five ectodermal parts were attached separately as small cones. On the following days the ectodermal components gradually covered the surface of the endo-mesodermal hemisphere, and four of them fused with each other without losing their original individuality. Twelve days after the operation (culture temperature 21°–26° C.), when the large combined explant was fixed, it was found attached to the glass bottom with a cylinder of naked endoderm from which protruded a somewhat irregular, elongated body. It was covered completely with the epidermis, half-transparent and expanded. The size of the explant: 1.66 mm. × 1.28 mm.

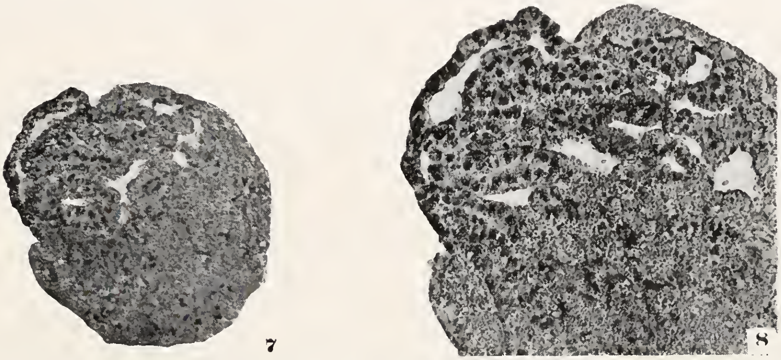


FIGURES 5 AND 6. Development of the isolated ventral marginal zone. Fig. 5. Differentiation of blood island (in the center), enclosed in the mesothelium, externally covered by the epidermis. Fig. 6. Blood cells, blood vessels, and mesenchyme besides epidermal covering and undifferentiated endoderm. × ca. 60.

Microscopical examination reveals that the protruded part of the explant is filled with loose mesodermal tissues: the yolk-rich mesenchyme, blood vessels enclosing blood cells and the mesothelium lining a wide space which is filled with blood cells (Fig. 6). Within the meshwork of mesenchyme and blood vessels is differentiated a piece of pronephric tubule, with one end attached to the endodermal cylinder.

VRK 5. From two gastrulae of stage II the medio-ventral sectors were cut out, fused together and cultured in Holtfreter solution. In the course of two days the explant formed a smaller compact sphere attached loosely to the bottom of the culture dish. Cultured for fifteen days at 18°–23° C., the size of the explant: 0.84 × 0.78 mm.

Microscopical study shows that ca. three-quarters of the external surface of the explant is covered by endodermal epithelium. The latter is replaced by a well-differentiated mesodermal epithelium on the remaining surface. Within the mesodermal epithelium a cavity is formed which encloses a large amount of highly entangled pronephric tubules (Fig. 7). The tubules are connected with the external epithelium at some points. There exists no clear boundary between the tubules and epithelium and they both show a similar histological construction (Fig. 8).



FIGURES 7 AND 8. Differentiation of nephric tubules out of the isolated ventral marginal zone. A quantity of yolk-rich endoderm, no ectodermal component. Fig. 7, × ca. 60; Fig. 8, × ca. 100.

Obviously the external epithelium is nothing but the pronephric tissue modified by the topographical relation. Some sections of the tubules are free of yolk and better differentiated, while the others are laden with much yolk and seem to be retarded in differentiation. In all sections the central canal is poorly developed. A small quantity of mesenchyme, blood cells and melanophores are differentiated among the tubules. Embedded in the endoderm are some concentrated groups of mesoderm cells without any clear differentiation.

VRK a'. Operated as in the last example and cultured for eleven days at 19°–22° C. The explant developed into a spherical vesicle covered by the epidermis. Later two protuberances were formed at each pole, one elongated, the other spheroidal in shape. The size of the explant: 1.24 × 0.73 mm.

Microscopical study reveals that the main part of the highly expanded explant is filled with a large amount of blood cells enclosed in a cavity lined with mesothelium (Fig. 5). In the narrow space between the mesothelium and the epidermis are encountered a smaller quantity of blood cells, blood vessels, mesenchyme and a few melanophores. Each of the above-mentioned protuberances contains a part consisting of endodermal cells without any histogenesis.

The behavior of the explants as observed in the living condition may be summarized as follows. During the first several hours after isolation the explant acquired a more or less spherical form with expulsion of a small number of free cells. In the subsequent one to two days the pigmented ectodermal part of the explant became separated from the endo-mesodermal part by developing a prominence which exhibited form changes of considerable degree. Thereafter the prominence retreated and the ectodermal part gradually fused to the endo-mesodermal ball, covering it more or less perfectly. In some cases the ectodermal prominence was constricted away from the endo-mesodermal part. Further morphogenesis of the explant is variable. It may develop a spheroidal compact body as in the case of the above described *VRK 5*, or a free spheroidal body covered by the epidermis as in the case of *VRK a′*, or in still other cases, a flat discoidal body firmly attached to the bottom and either covered or not covered by the epidermis. After the establishment of any of these forms further change in the external form was infrequent, except the enlargement through uptake of water from without, which often resulted in more or less transparent explants. In one case a minute tail-like formation developed from a discoidal explant (see below).

The results of the histological study of seventy-nine available cases are summarized in Table I. The explanted mesoderm has developed the following tissues: blood cells, pronephric tubules, mesothelium, blood vessels, mesenchyme and possibly melanophores. The table shows clearly that no significant difference can be recognized in the frequency of these tissues between series IIa and IIb (Table II). Therefore we can neglect for the moment this difference in operation and treat IIa and IIb together.

TABLE I

Differentiation of the isolated ventral marginal zone

Origin of explants	Number of explants	Frequency of tissues differentiated in the explants							
		Blood cells	Mesothelium	Blood vessels	Mesenchyme I	Mesenchyme II	Melanophores	Nephric tubules	"Somite"
Lateroventral (IIa)	44	34 77%	11 25%	9 20%	4 9%	3 6%	8 18%	25 56%	2 4%
Medioventral (IIb)	35	22 62%	6 17%	7 20%	7 20%	1 2%	1 2%	20 57%	1 2%
Total	79	56 70%	17 21%	16 20%	11 13%	4 5%	9 11%	45 56%	3 3%

Blood cells. In most cases these occur in large numbers in a condensed group comparable to the normal blood island. In eighteen cases the blood cells are found in a voluminous cavity lined with mesothelium (Fig. 5). In twenty-four cases they are differentiated in a restricted space developed within the endoderm, or between the endoderm and the bottom of the culture dish. In fourteen other cases blood cells are gathered in a smaller group between mesenchyme or nephric tubules.

Nephric tubules. Tubules occur in the majority of cases in a restricted space between endoderm mass, or completely embedded in the latter, or (rarely) in the mesh-work of the mesenchyme and blood vessels. When they are perfectly embedded in the endoderm their histogenesis is very poor, but when they are bathed in the fluid of a cavity and invested with mesenchyme and blood vessels their histogenesis is more typical.

TABLE II

Significance of the origin of explants (medio-ventral or latero-ventral) and the number of pieces fused together for the differentiation of explants from the marginal zone

Origin of explants	Number of pieces fused together	Number of explants	Principal differentiations		
			Blood cells	Nephric tubules	"Somite"
Lateroventral (IIa)	2	44	34 77%	25 56%	2 4%
Medioventral (IIb)	1–2	26	15 58%	15 58%	1 3%
	5–6	9	7 77%	5 55%	0
Total		79	56 70%	45 56%	3 3%

It is interesting to note that between the blood cells and tubules the following relation exists:

(a) explants with blood cells but without tubules........................34 cases (43%)
(b) explants with tubules but without blood cells........................23 cases (29%)
(c) explants with both of them..22 cases (27%)

Thus, all the explants have developed either one or both structures. The blood cells and tubules can be considered, therefore, as the main differentiations of the explants.

Mesothelium. A thin epithelium, lining rather voluminous cavities and developed within the epidermis, is considered under this heading. It is accompanied in most cases by blood cells, and often by blood vessels. In sections through explants with such a cavity, a colloidal constituent is precipitated in the form of finer granules or reticulum, which is stained intensively with "Blauschwarz." Quite probably the cavity formation is the result of the release of colloid from the mesothelium cells or mesenchyme cells.

Mesenchyme. Under mesenchyme two defined groups are encountered. One is characterized by large, yolk-laden cytoplasm and a very coarse mesh-work which is stained only slightly with "Blauschwarz" ("mesenchyme I" in Table I). The other is characterized by the yolk-free cytoplasm and slender processes intensively stained with "Blauschwarz" ("mesenchyme II" in Table I). In one explant in which the limb-bud is developed, mesenchyme II occurs in large quantities. Type I is far more frequent than type II and is observed mostly in association with blood vessels and blood cells.

Blood vessel. Blood vessels are formed either within the mesh-work of mesenchyme (Fig. 6) or just beside the mesothelium. Apparently they do not form a perfectly closed system. Blood cells are not always found within the vessels.

Melanophores. Out of nine explants with melanophores three do not possess an ectodermal component. In these cases, at least, it is probable that the melanophores are derived from the marginal zone cells and not induced from the ectoderm as a mesectodermal component.

Myoblasts. In a few cases a small part of the explanted mesoderm gave rise to a group of elongated cells which have the features of a myoblast. But in not a single case are definitive muscle cells with myofibrils differentiated. More often, condensed mesodermal cells are found in connection with the pronephric tubules. They may be considered as a fragment of an early somite before the myotome is segregated from another part of it. However, the differentiation did not progress further and we cannot designate these cells even as myoblasts.

Tail-like process. The above-mentioned minute process which occurred only in one explant, reveals itself in section as a very small tail-like process filled with mesenchyme II. No axial structures are found in it. But proximal to the process three small groups of mesoderm cells are found. One of them is composed of mesenchyme cells which are in the process of breaking up into free cells (of type II). Two remaining groups are compact but have apparently the same fate as the first. In none of the three groups is a differentiation of myoblasts discernible. Taken as a whole, the structure may be considered an incomplete tail without axial tissue. In other parts of this explant the mesoderm has differentiated nephric tubules and a small amount of blood cells.

Summarizing the observations of this series, it may be said that both the isolated medio-ventral and latero-ventral sectors of the marginal zone differentiate in the main: blood islands, nephric tubules and derivatives of the lateral plate. But in a very restricted number of cases (3.7 per cent) and in restricted quantity, some features of somitic development are observed, in no case, however, leading to complete differentiation of a myotome. Not a single explant differentiated notochord or definite muscle cells.

Table II shows further that the difference in the number of the pieces fused together did not have an important effect on the outcome of the differentiation so far as the present investigation is concerned. But from these restricted results it is not permissible to draw any general conclusions as to the relation between the quantity of the material and the quality of differentiation.

Comparing the differentiation of the explant with its prospective value, the following may be pointed out. The differentiation of blood island, mesothelium, blood vessels and probably also of mesenchyme I corresponds to the prospective value of the isolated region. But it is rather questionable whether the nephric tubules are contained in the prospective value of this section of the marginal zone, as maintained earlier by Holtfreter (1938). The somitic development which occurs very seldom and incompletely is surely not in accord with the prospective value of the isolate. Thus we must conclude that the main feature of development of the explanted ventral marginal zone corresponds to its prospective value, although there is a recognizable tendency to differentiate structures which lie more dorsally in the dorso-ventral scale of trunk mesoderm differentiation than the prospective value of the material.

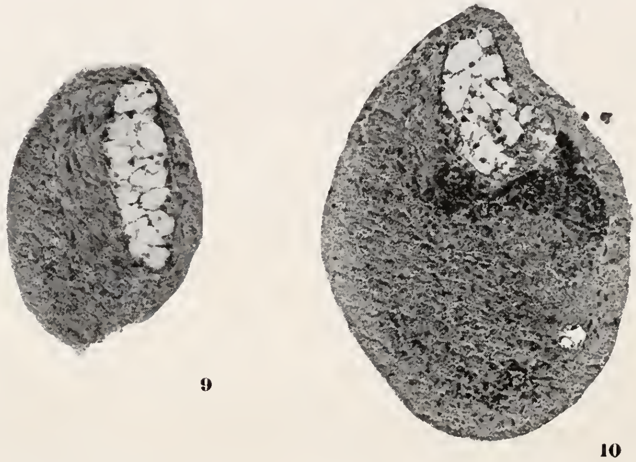
Experimental Series: Shock Treatment of the Isolated Ventral Marginal Zone with Ammonia

In this series only the medio-ventral sector of the marginal zone as defined above (Fig. 4) was used as the material. The isolate, prepared in the same way as in the last series, was treated with 0.02 per cent ammonia solution until the disintegration of the isolate took place, then transferred to Holtfreter solution where a part of the cells reaggregated. In this case more time was required for the disintegration than in control series I. This is perhaps due to the presence of a thick layer of yolk cells within the isolate, which are apparently more resistant to the dispersive action of the medium and protect the superficial layer of mesoderm and ectoderm from the action. An attempt was made to stop the treatment at a state of disintegration, which was comparable to that attained in the control series I. This was realized, however, only approximately, as the variability in the velocity and detailed course of the disintegration process was unexpectedly high. The period of treatment, which was determined according to the morphological state of disintegration, varied between 3½ to 7 minutes. One aggregate was obtained from a single original isolate, or from a number of original isolates. In the latter case the original isolates were treated with ammonia and the disintegrating masses were fused into one mass with the aid of a glass needle and transferred to a dish containing Holtfreter solution. Without further manipulation the cells reaggregated into a single body. In this way, two to ten original isolates were fused into a single aggregate. However, it must be kept in mind that the quantity of the resulting explant is much smaller than the sum of the original isolates, as a severe loss of cells occurs during and after treatment.

The morphological aspect of the course of disintegration of the marginal zone in ammonia solution, differed from that of the ectoderm in that the curling was insignificant and the process of actual disintegration was foreshadowed by a slow but steady rise in transparency of the cells. Some examples follow:

AVR 92. From four gastrulae of stage I, the medio-ventral piece of the marginal zone was isolated and treated with 0.02 per cent ammonia solution for ca. 4 minutes until the original contour of the individual isolates was almost lost, then transferred to Holtfreter solution. A severe loss of cells was observed at the time of treatment and during subsequent days, resulting in reduction of the size of aggregates. Large white endoderm cells, light brown mesoderm cells and dark pigmented ectoderm cells were discharged. Five days after the treatment the ovoid aggregate showed a small dark groove on one of its poles, while the rest of the surface was covered by the opaque, white endoderm. The explant adhered loosely to the bottom of the culture dish. Cultured for fifteen days at a temperature of 14°–24° C., the size of the explant: 1.81 × 0.90 mm.

Microscopic observations: Covered by an undifferentiated endoderm layer, a large complex of notochord and muscle developed (Figs. 9, 10). In one-half of the explant the notochord is only slightly curved and approximates the normal notochord of the corresponding stage in the diameter (0.15 mm.), while in the other half the notochord takes an irregular complex course and shows varying cross sections. Most of the notochord is composed of perfectly vacuolated cells and provided with a layer of connective tissue cells. The muscle tissue which accompanies the notochord is regularly segmented where the course of the notochord is more or less straight, but irregularly arranged where the course of the latter is complicated. The muscle tissue is provided with clearly differentiated myofibrils and a small amount of yolk granules, and invested with a layer of connective tissue cells, which seem to be derived from the original somite. Some elongated neural bodies are formed among the rather irregular complex of notochord and muscle. In some of its parts the neural tissue is laden with exceptionally abundant yolk granules, and does not have a definite boundary against the neighboring mesoderm. It is difficult to identify the neural structure as any part of the normal nervous system, but as it is stretched in a slender form one can perhaps ascribe to it a caudal nature. A small group of the pronephric tubules are formed between muscle, notochord and endoderm. A mesodermal protuberance exposed to the medium is formed. This looks like a limb-bud, but is partly continuous with a fragment of neural tissue.

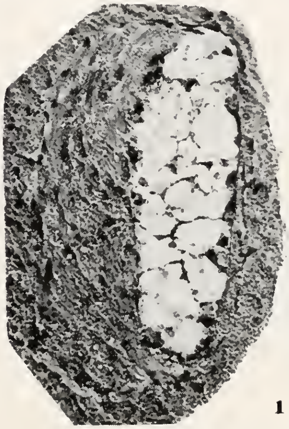


FIGURES 9 AND 10. Differentiation of notochord, muscle and neural tissue from the isolated ventral marginal zone after exposure to ammonia solution. Two sections through the same explant. Fig. 9. Notochord (right) and muscle (left) enclosed in the endoderm. Fig. 10. Somewhat irregular notochord accompanied by the muscle (left) and neural tissue (below). × ca. 60.

AVR 152. Three pieces of the medio-ventral section of the marginal zone were isolated from gastrulae of stages I and II and treated with ammonia solution for ca. 3½ minutes. Before the disintegration set in they were put back into Holtfreter solution. The aggregate formed a somewhat deformed sphere with small pigmented cell-groups protruding from its otherwise smooth whitish surface. An appreciable quantity of cells were discharged. Cultured eight days at 22°–24° C., the size of explant: 1.40 × 0.65 mm.

Sections through the explant show that the center of the ovoid explant is occupied by a piece of notochord with very irregular contour (Fig. 13). The maximal diameter is found to be 0.17 mm., a value which exceeds the value of a normal notochord of the corresponding stage. Some of the notochord cells are in an earlier phase of the vacuolization showing numerous small vacuoles within the cytoplasm, while others are completely vacuolated, retaining a minimal amount of cytoplasm. The notochord is incompletely surrounded by myotomes. In the periphery of each myoblast, the myofibrils are differentiated and enclose internally a good quantity of yolk granules. In some parts of the explant both tissues, the notochord and myotome, are not perfectly delimited, having a transitional zone in which the cells show an intermediate character. To one end of the notochord is attached a piece of neural tissue which is devoid of a definite structure, and is laden with abundant yolk. Some of the myotomes are connected with this neural tissue without clear boundary. The external covering of the explant is chiefly a layer of endoderm.

At the pointed end of the explant the covering is ruptured by the above mentioned neural tissue with one extremity of the notochord embedded in it. The myotomes are directly applied to the endodermal covering, except in the region where a group of nephric tubules are formed between them. No blood island or mesothelium is differentiated.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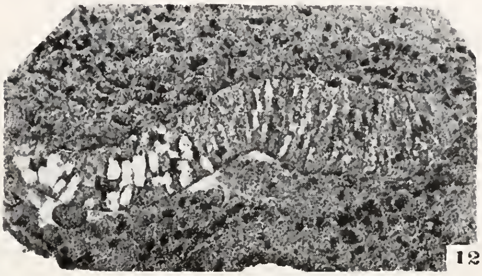
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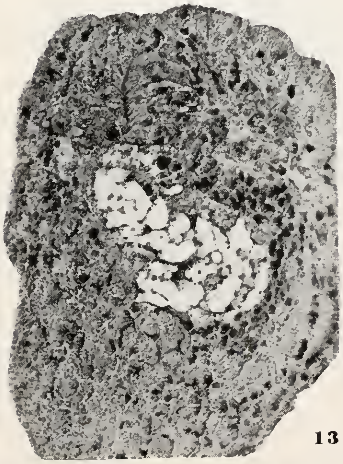
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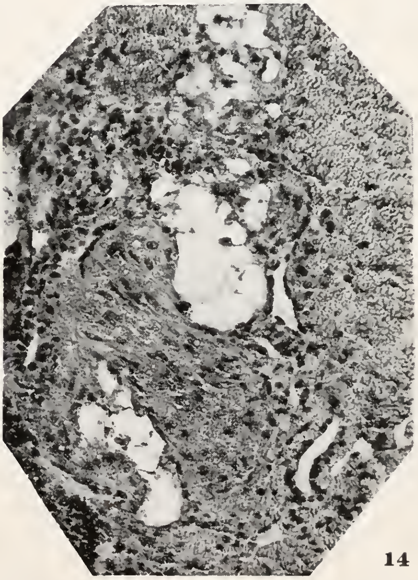
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FIGURES 11–14. Different types of notochord induced in the ventral marginal zone through ammonia-treatment. Fig. 11. A straight cord composed of perfectly vacuolized cells, accompanied by myotomes on the left side. Fig. 12. A typical young notochord, in which the vacuolization has progressed farther toward one end (left in the picture) than toward the other. Fig. 13. Rather atypical differentiation of the notochord, not clearly delimited from the surrounding myotomes (above and below) and neural cells (right). Fig. 14. In the upper third of the figure notochord cells are scattered within a field of neuralized cells and myoblasts. In the lower two-thirds an entangled notochord is formed within the muscle tissue. At right, below nephric tubules. All figures × ca. 100.

AVR 32. Three pieces of the medio-ventral marginal zone were isolated from gastrulae of stage I and immersed in ammonia solution. The treatment was continued until the isolates began to disintegrate into separate cells (ca. 5 minutes). In Holtfreter solution, the cells aggregated into an elongated, compact group which, in the course of cultivation, adhered loosely to the bottom. A rather small number of cells were set free. When the aggregate was fixed in Bouin's fluid after thirteen days of culture at a very low temperature (12°–22° C.), it was covered by a whitish layer of endoderm, through which an elongated dark field was vaguely visible. The size of explant: 1.78 × 0.89 mm.

Microscopic observations: A large piece of notochord (length ca. 1.2 mm.) runs along the long axis of the explant. Consistent with the low temperature it shows the first sign of its histogenesis. One end of this piece, which may be called "caudal" because of its delayed histogenesis, is twisted in a spiral, with its highly flattened, yolk-laden cells arranged in a single row like a pile of coins. The cytoplasm is almost devoid of vacuoles. The middle part of the piece is almost straight (Fig. 12). The arrangement of flattened cells in a single row is very accentuated and regular. Small vacuoles are scattered at random throughout the cytoplasm. In the "cephalic" third of the notochord the vacuolization has progressed further. Vacuoles are enlarging, cells are consequently expanding and are not as flattened as in other parts (Fig. 11). The nucleus is polygonal in cross section, being enclosed in a larger vacuole. The diameter of the notochord equals that of the normal trunk notochord at the corresponding stage (0.11 mm.). The notochord is surrounded on all sides by the mesoderm, which shows at some points the first sign of myotome formation. Near each end of the notochord a small cavity is formed lined by a columnar epithelium with a pigmented surface. They seem to represent the neural tubes. Both tubes are at many points continuous with the adjoining mesoderm, which is developing into myotomes. Moreover, at the "caudal" end the notochord is directly continuous with the wall of the neural tube. An endoderm layer with an uneven surface covers all these structures.

Observations during the cultivation may be summarized as follows. A large number of cells were discharged just after the treatment and also during the first three days of cultivation. Not only smaller ectodermal and mesodermal cells, but also large yolk cells were found among the free discharged cells. After the recovery, the external surface of the aggregate was mostly covered by the whitish endoderm layer. The ectoderm, if present, withdrew into the interior, often leaving a small neuralized fragment on the surface, except in several cases where the epidermis covered one part of the explant. As a rule, the mesoderm occupied the interior of the explant. The form change during the subsequent cultivation was

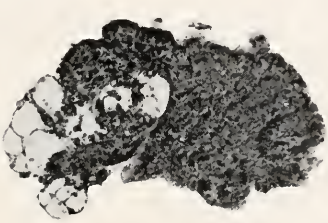
TABLE III

Differentiation of the isolated medio-ventral marginal zone treated with ammonia

Number of explants	Frequency of rudiments and tissues differentiated in the explants									
	Notochord	Muscle	Myoblasts	Limb-bud	Nephric tubules	Mesenchyme 11	Melanophores	Blood cells	Neural tissue	Otic vesicle
40	30 75%	25 62%	16 40%	2 5%	29 72%	10 25%	8 20%	2 5%	30 75%	2 5%

pronounced only in three cases where the tail developed from the explant, covered partly by the epidermis. A much less pronounced protuberance often developed on the surface covered by the endoderm. The free spherical, half-transparent body covered by the epidermis, often observed in control series II, did not occur. The explants were cultured for eight to seventeen days at room temperature before fixation in Bouin's fluid.

The results of the microscopic examination are summarized in Table III. Notochords obtained here have a varied appearance. Of thirty cases with notochordal differentiation, eight show only a small number of scattered vacuolated cells among the myotomes, while in other cases (twenty-two explants, 55 per cent of all available cases) a more or less defined notochord is differentiated. Of these cases six show an almost typical notochord with the diameter corresponding to that of a normal one. Very often (sixteen cases) the notochord occurs in an irregularly twisted form or in an aberrant form. These are found among neural tissue, myotomes or undifferentiated endoderm, exhibiting a great variation in the size of each vacuolated cell. Often cell types, which are intermediate between notochord, neural tissue and myotome, accompany these atypically formed notochords (Fig. 14). In only two cases the induced notochord developed a tail with accompanying neural tube and myotomes (Fig. 17); while in the other cases one might ascribe to the notochord the trunk character, since it possesses a larger diameter and does not show any tendency of pronounced stretching (Fig. 16). In most cases the notochord occurs within a covering tissue, but it may be exposed to the medium in the form of an unorganized tissue fragment (Fig. 15).



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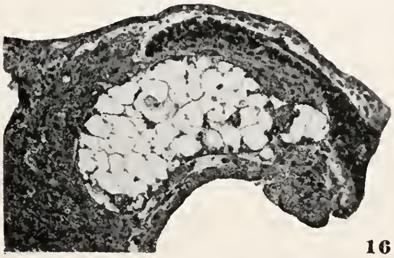

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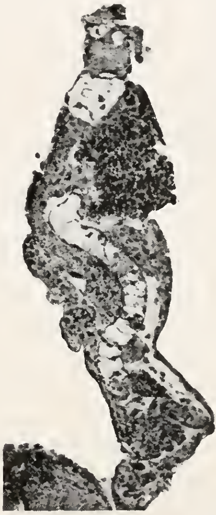

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Figure 15. Uncovered notochord from the isolated ventral marginal zone treated with ammonia, accompanied by neural tissue and muscle cells.

Figure 16. A large notochord with myotome and spinal cord, induced with ammonia-treatment. The upper side of the explant is covered by the epidermis, the lower side with endoderm.

Figure 17. A tail-like structure developed from the explant, shown in Figure 16. A spiral notochord, with irregular mass of the myotome and neural tissue. All figures × ca. 60.

With a few minor exceptions the notochord is accompanied by myotomes and neural tissue. The myotomes attain a good differentiation and typical arrangement only when a typically formed notochord exists in their direct vicinity. The neural tissue and notochord have also an intimate relation. Often the fragments of notochord tissues are embedded in the neural tissue (Fig. 15) or are enclosed by a neural epithelium. Also, in the other cases, both tissues have the tendency to occur in association. Only two explants belonging to the group of imperfect notochordal differentiation are devoid of neural tissue. Here only a small number of notochord cells are scattered among the myotomes.

Neural tissue is formed very frequently (in thirty out of forty cases or 75 per cent). In twenty cases, it is differentiated on the surface of the explant without any characterization and cannot be identified with any part of the normal nervous system. The same holds for the aberrant neural tissues developed inside the explants (six cases). Only in three cases is a fragment of the spinal cord differentiated which runs along the notochord (Fig. 16). In two cases the neural cord is found as a component of the tail. Their morphogenesis is rather atypical. Differentiation of a brain cannot be ascertained in any explant. In two cases a structure developed which resembles the otic vesicle. One of these is accompanied by two ganglion-like cell groups. No nasal placode or lens is observed. Most of the neural tissues are partly or wholly laden with an exceptionally large amount of yolk granules. A remarkable consequence of this is the occurrence of clearly differentiated neurofibrils just beside the dense group of yolk granules. In younger explants the neural tissue and mesoderm have no clear limiting surface and one gets the impression that both tissues are derived from a common material.

A myotomal differentiation is found in thirty-nine of forty explants (97 per cent). Of them, twenty-five show the muscle cells with myofibrils, while other explants contain only myoblasts without fibrils. In at least five of the latter cases the explant was fixed too early for fibril differentiation to have occurred. The myofibrils exhibit the best differentiation when a normally formed notochord runs in the vicinity of the myotome. In these cases the parallelism between the course of the notochord and muscle cells is very conspicuous. Aberrant formation of the notochord is paralleled by disturbed arrangement of the muscle cells. There exists an almost perfect correlation between the occurrence of notochord and the differentiation of the myofibrils: (1) If the notochord is absent, fibrils are not differentiated (five cases); (2) In the presence of scattered notochordal cells, half of the explants differentiated fibrils (ten cases); (3) In the presence of a well defined notochord the fibrils are always differentiated (nineteen cases, excluding three prematurely fixed cases). A limb-bud is formed only in two explants. One of them shows an extensive proliferation of mesenchyme cells (type II). The nephric tubules are developed in twenty-nine out of forty cases. They are better differentiated when the explant contains notochord and muscle as well. A corresponding situation was found by the author in isolated mesoderm from the neurula. This influence of the axial organs on the development of the nephric system deserves further analysis. The blood island is found only in one case, in a restricted cavity between the endoderm and bottom of the culture dish. In no case did a cavity, lined with the mesothelium or filled with yolk-rich mesenchyme and blood vessels, develop in the explant.

Since in this series the number of the explants fused together varies considerably, it seems advisable to inquire whether the frequency of the notochord differentiation depends upon the number of fused pieces. In Table IV, the relation between the notochord differentiation and the number of original pieces is summarized. It appears that the notochord can develop in the explants from one isolate as well as in those from ten isolates. No significant difference in notochord frequency seems to exist between groups of one to three isolates.[2] It is probable that in explants with more than four isolates the frequency is higher, although the small number of cases does not allow any definite conclusion. On the other hand, the morphogenesis of the induced notochord may be influenced in some degree by the size of the explant. In explants from one isolate the notochord cells occur in small scattered groups without forming a cord-like tissue, while in explants from more than two isolates a more or less typically organized notochord can be developed.

TABLE IV

Relation between the number of the original isolates fused together and the frequency of notochord differentiation

Number of isolates fused together	Number of cases	Explants with notochord differentiation	Frequency of notochord in %
1	14	10	71
2	11	8	72
3	12	9	75
4	1	1	(100)
6	1	1	(100)
10	1	1	(100)
Total	40	30	

Results of this series of experiments may be summarized as follows. The isolated, medio-ventral sector of the marginal zone of the earlier gastrula together with a small amount of adjacent ectoderm and endoderm, differentiates notochord, muscle and nephric tubules as well as neural tissues, if it is briefly exposed to an ammonia solution and then cultured in Holtfreter solution.

DISCUSSION OF RESULTS

An appropriate understanding of the results of control series I may be possible only when the behavior of the untreated explant of the ectoderm of a gastrula of *Triturus pyrrhogaster* in Holtfreter solution is known. Such explants differentiate into nothing but atypical epidermis. This result, confirmed repeatedly by the author and described in another paper in preparation, shows clearly that the isolated ectoderm of this species behaves like that of *Triturus torosus* and does not respond to isolation and cultivation by undergoing neural differentiation as does the isolated ectoderm of *Amblystoma punctatum.* Hence the neural differentiation obtained in control series I must be ascribed to the ammonia treatment. Study of the induced structures in this series has shown that they have more or less distinct characteristics of cephalic ectodermal rudiments and tissues such as brain, eye, nasal placode, lens, mesenchyme, melanophores and frontal gland. In another paper will be presented data which show that comparable differentiations occur in the isolated ectoderm induced by a multitude of substances and also by a living cephalic organizer; the similarities between these induced structures and corresponding normal structures cannot be a mere coincidence. The conclusions conform in general with those obtained by Holtfreter (1944a, 1945, 1947) in so far as the "self-induced" explants of*Amblystoma punctatum* and the imperfectly cytolyzed explants of *Triturus torosus* are concerned.

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[2] The significance of the difference in frequency of the notochord in the explants from one isolate and in the explants from three isolates in the above table, was tested after the exact treatment of R. A. Fisher and the probability of occurrence of the observed set of frequency was found to be 0.795. Hence we *cannot* conclude from the above data that notochord occurs in the explants from three isolates more frequently than in the explants from one isolate.

Comparing the results obtained in control series II and the experimental series it seems likely that the ammonia-treatment induces in the isolated medio-ventral marginal zone the differentiation of notochord and muscle, and suppresses the development of blood cells and mesothelium. But before this can be concluded we must exclude two possible sources of error: (1) *The development of dorsal tissues may be caused by the presence of some cells of the adjacent marginal zone taken along erroneously at the operation.* This possibility seems to be excluded, because in control series II it was shown that even the latero-ventral section never gave rise to notochord and muscle. It may be remembered that the dorsal limiting line of this section lies 10° more dorsal than that of the medio-ventral section, which was exclusively isolated in the experimental series (Fig. 4). (2) *The differentiation of the dorsal tissues may be brought about by the volume increase following the reaggregation of cells from many isolates into a single aggregate.* We have shown above that axial differentiation is possible even in the ammonia-treated explant originating from one isolate and having a smaller quantity than the average explant of control series II. On the other hand, in control series II, typical differentiation of the ventral mesoderm occurred even in the explants originating from six pieces of medio-ventral section. The second possible source of error can also be rejected.

Thus, it may be concluded that through ammonia-treatment the mode of differentiation of the isolated medio-ventral marginal zone can be altered, so that ventral mesodermal differentiations such as the blood island, blood vessels and mesothelium are suppressed, whereas dorsal differentiations such as the notochord and muscle are initiated, and intermediate differentiations such as the nephric tubules are not significantly altered (Table V).

The close parallelism and intimate spatial relation between the notochord and neural tissues observed in the experimental series deserves special attention. This correlation can be most readily explained if we assume that the neural tissue is induced in the ectodermal component of the explant by that part of the mesoderm which later differentiates into notochord and muscle. However, it must be recognized that the following possibilities are by no means excluded: (a) The neural differentiation and notochordal differentiation are induced separately in the ectodermal and mesodermal component respectively by the ammonia-treatment. The observed spatial relation between both tissues results from a later mutual approach and fusion. That such mutual approach occurs between tissues of the same nature is abundantly exemplified (Yamada, 1937; Holtfreter, 1944b) and it is not improbable that an analogous attraction also exists between different tissues of the

TABLE V

Comparison of the mode of differentiation of the ammonia-treated and non-treated isolate from the ventral marginal zone

Differentiation	Frequency of tissues in % Control series II (non-treated)	Experimental series (ammonia-treated)
Notochord	0	75%
Muscle	0	62%
Mesenchyme II	5%	25%
Melanophores	11%	20%
Nephric tubules	56%	72%
Blood cells	70%	5%
Blood vessels	20%	0
Mesothelium	21%	0

axial region. (b) One part of the neural tissue obtained did not originate from the ectodermal component but from that part of the mesoderm which otherwise became notochord and muscle. Holtfreter (1938) showed that the dorsal marginal zone has the tendency to differentiate neural and other ectodermal tissues, besides notochord and mesodermal tissues, if it is isolated and cultured in Holtfreter solution. If, in the present case, ammonia-treatment makes the ventral marginal zone equivalent to the dorsal marginal zone, it is very probable that the same process can occur in these explants. Indeed, the morphogenesis of the ammonia-treated isolate of the ventral marginal zone does not differ much from that of the isolated organizer. As repeatedly pointed out above, a part of the neural tissue obtained in the explant shows intimate connections with the mesoderm and often a very high content of yolk granules. These facts can be most readily explained if we assume these possibilities: (c) One part of the obtained notochord did not originate from the mesodermal component but from the ectodermal component of the isolate, which was otherwise neuralized. This is not impossible since the presumptive ectoderm can be induced to form notochord and muscle, if it is applied to the trunk mesoderm of the neurula (Holtfreter, 1933). (d) The differentiation of the notochord was evoked in the mesoderm by the presence of the ectoderm which was neuralized by the treatment. This possibility might seem to contradict the observed fact that the neuralized tissue forms (in control series I) rudiments of more or less distinctive cephalic type, while in the experimental series the neuralized tissue shows characteristics of trunk and tail. This fact apparently supports the first assumption that the neural tissue is induced secondarily by the notochord and mesoderm. In spite of this we must not completely neglect the possibility that the neuralized ectoderm acts as an inducer on the mesoderm, while the subsequent differentiation of the ectoderm is inversely governed by the mesoderm or notochord and, consequently, the neural tissue acquires a trunk or tail nature (Yamada, 1939).

It is probable that many of these possibilities are realized in our experiments. An experimental test of these possibilities is not as simple as one might expect, as the boundary between the ectoderm and mesoderm is very indistinct on the surface of the early gastrula, and some observations suggest that the induced differentiation of the notochord is connected with this boundary. This problem will be the subject of a further series of experiments.

Theoretical Considerations

In order to discuss the theoretical significance of the present results, it seems necessary to introduce the double-potential theory proposed by the writer in an article written in Japanese (Yamada, 1947), as it furnishes the basis for the present investigation.

The essential points of the theory can be described most concisely as follows: (1) The developmental activity of a given germ region depends, above all, upon two sorts of "potentials,"[3] which are designated respectively the dorso-ventral and cephalo-caudal, and can vary relatively independently of each other. (2) At a given developmental stage, the dorso-ventral and cephalo-caudal potentials show a definite gradient along the respective axis; or, otherwise expressed, every germ region has a definite value of both potentials according to its topographical position relative to both axes. (3) In the course of development the absolute values of both potentials show a continual change, which is characteristic for the germ region and for the stage. Thus the developmental activity of any germ region may be defined as the combined effects of the time-curves of both potentials. (4) The change in potential is effected partly through the factors inherent in the germ region and partly through factors external to it. The latter factors are called the mediators. There are two of them . . . the dorso-ventral and cephalo-caudal mediators affecting respectively the dorso-ventral and cephalo-caudal potentials.

This theoretical scheme is constructed to deal with the early ontogenesis of amphibians, but it is expected that the same principle can be adapted to other developmental systems, with alterations of unessential points.

To apply this scheme to the present problem, we have to follow some suggestions made earlier: (a) Considering the analysis of the normal course of development and the experimental data, it was assumed that the dorso-ventral potential is connected primarily with the intensity of the biochemical activity of the developing system, while the cephalo-caudal potential is connected primarily with the morphogenetic movements of the developing system. (b) Most of the phenomena, called embryonic induction, involve a shift of the dorso-ventral potential toward the dorsal end, and may be in this sense called dorsalization. The dorso-ventral mediator, which is responsible for this effect, seems to be (in most cases) chemical in nature. Many chemical substances or procedures can play the role of a dorso-ventral mediator. (c) The morphogenetic indifferent state, such as is found in the presumptive ectoderm of the early gastrula of many amphibians, is characterized by the ventral (low) value of the dorso-ventral potential and the cephalic (low) value of the cephalo-caudal potential. This means that through the action of any dorso-ventral mediator, such a germ region can be induced to form cephalic axial organs (eye, brain and nose), while without such action of mediators this germ region will differentiate only epidermis in the urodeles and epidermis and sucker (the cephalic and ventral differentiation) in the anurans. If we want to get caudal axial organs out of the same material, the cephalo-caudal mediator must be applied as well as the dorso-ventral mediator, to shift the cephalo-caudal potential in caudal direction. The ventral value of the dorso-ventral potential and the cephalic value of the cephalo-caudal potential being called "low," the effect of the dorso-ventral and cephalo-caudal mediator can be designated, in this case, as a "rise" in respective potential value. (d) If a germ region with a high value of one of the potentials comes in contact with a germ region with a low value of the same potential, the former generally acts as the mediator, provided that an appropriate contact surface is established. The organizer acts through the coordinated spatio-temporal pattern of both mediators, which is found in the potential composition of the germ region concerned, i.e., the dorsal marginal zone.

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[3] The concept of "morphogenetic potential" was first introduced by Dalcq and Pasteels (1937) in their theory of morphogenesis. As will be clear from the following text, in our theory a meaning is given to this concept which differs much from that in the theory of the Belgian authors. However their basic contributions to the theoretical problem here treated must be appreciated.

From the standpoint of the double-potential theory the morphogenetic effect of sublethal cytolysis can be designated as dorsalization; in other words it raises the dorso-ventral potential of the developing system. If the presumptive ectoderm of the early gastrula, with low values of both dorso-ventral potential (*Pdv*) and cephalo-caudal potential (*Pcc*), is treated with a sublethal dose of the cytolyzing agent, the *Pdv* will be raised to a higher value while the *Pcc* will be left unchanged. The end result must be a dorsal cephalic differentiation (such as brain and eye) without additional caudal differentiations (ear vesicle, spinal cord, notochord and muscle). The facts correspond well to this expectation. One of the most striking and, until now, unexplained features of the "self-induced" ectoderm is that it comprises almost exclusively structures of the foremost region of the head (Holtfreter, 1948) and is thus just what is to be expected from our theory.[4] The qualitative difference within the obtained cephalic inductions may be attributed, by this theory, to the difference of *Pdv*-curve and its spatial distribution in each case. A sharp rise of the *Pdv*-curve leading to the maximal value may be followed by differentiation of the eye. A less pronounced elevation may lead to the brain. A still less pronounced one corresponds to the nose-type, and a weak one, perhaps, to the mesenchyme and chromatophores. A rise of *Pdv*, in a small circumscribed area, may cause the differentiation of the lentoid.

It must be admitted that such a presentation neglects the normal elaboration of the organ rudiments. If we distinguish two phases in the development of an organ-rudiment from the germ layer, *viz.*, the phase of determination of the basic pattern of the organ, and the phase of elaboration of this pattern, the above discussion deals only with the former. For the latter we must perhaps take other factors into account, which need not be specific. In the isolate from earlier developmental stages the phase of elaboration seems to be absent, and the structures developed within it are, in no case, normally elaborated. As this imperfection of elaboration occurs also in the isolate with the living organizer, we cannot ascribe it wholly to an inadequate nature of the primary inductor.

What, therefore, will be expected from the theory when the same treatment is applied to the ventral marginal zone of the early gastrula? This germ region can be characterized as having a low *Pdv* and an intermediate *Pcc*, or more precisely, as being endowed with the inherent capacity to raise its *Pcc* to an intermediate level, because it gives rise to the ventral differentiations of the trunk region in normal development and also in isolation (control series II). The ammonia-treatment must, in this case, change only *Pdv* leaving the *Pcc* unaffected. The results may be the maximal value of *Pdv* and an intermediate value of *Pcc*. This means the

[4] It may be added in this connection that the theory was proposed at the time when the works of Holtfreter (1944, 1945 and 1947) were unknown in Japan.

differentiation of the axial organs of the trunk. Our results (experimental series) fulfill the theoretical expectation. The regional differences of *Pdv* within the isolated mesoderm may account for the occurrence of different tissues side by side; the maximal value corresponding to the notochord, the next highest value to the muscle and the intermediate one to the pronephros. That a good differentiation of the myofibrils occurs only in the presence of a notochord may be interpreted by the assumption that this differentiation presupposes a gradual rise of *Pdv* in the later stages, caused by the notochord which plays the role of dorso-ventral mediator owing to its special histogenesis. But we must not neglect the possibility that stretching of the muscle, which is caused by the notochord, plays an important role in the formation of its fibrils.

It is well known that many physiological and histochemical reactions show clear dorso-ventral gradients which are superimposable upon each other. This type of gradient is, for instance, exhibited by cytochrome oxidase, sulfhydryl groups of protein, ribonucleic acid, alkaline phosphatase and acetalphosphatide. Recent studies make it probable that at least some of these reactions are bound to submicroscopic granules called microsomes (Brachet, 1949). Studying the effect of cytolysis on the behavior of cytoplasmic ribonucleic acid, J. Brachet (1946) finds that the basophilia of the cytoplasm of embryonic cells of amphibia markedly increase when exposed to an abnormal salt solution, and that this basophilic material can be digested away by a dilute solution of crystalline ribonuclease. Holtfreter (1948) reports that an exposure of isolated embryonic tissue to salt solution of high pH produces "appearance and precipitation of basophilic granules in the previously hyaline portion of the protoplasm." From these observations it seems probable that ammonia-treatment causes an increase of the basophilic granules in the ectoderm as well as in the mesoderm. If this is the case, then there exists a clear parallel between the concentration of basophilic granules and *Pdv* in experimental as well as in normal conditions. In both cases the dorsal mode of development occurs in the region with high granular content, and the ventral mode in the region with low content. This may mean that *Pdv* is nothing but the capacity of cells to maintain or produce a certain concentration of these granules. Recent data on the role of ribonucleic acid in many cytological elaborations and formation of fiber protein (Brachet, 1949), make it probable that differentiations of neurofibrils, myofibrils and notochord vacuoles (which occur exclusively in the region of high *Pdv*) are in some way connected with the basophilic granules.

SUMMARY

(1) Control series I. The ectoderm of the early gastrula of *Triturus pyrrhogaster* was isolated and exposed for a short time to a solution of ammonia and then cultured in Holtfreter solution. The isolate differentiated structures which can be characterized as cephalic ectodermal rudiments including brain, eye, nose and frontal glands. Otic vesicles, spinal cord and mesodermal structures were wholly absent.

(2) Control series II. The ventral marginal zone of the early gastrula was isolated and cultured in Holtfreter solution. It differentiated blood island, blood vessels and mesothelium and nephric tubules.

(3) Experimental series. The isolated ventral marginal zone was treated with ammonia solution and cultured in Holtfreter solution. It gave rise to notochord

and muscle as well as nephric tubules. The development of the blood island, mesothelium and blood vessels was almost completely suppressed.

(4) Results were interpreted from the standpoint of the double-potential theory (Yamada, 1947).

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SEROLOGICAL SYSTEMATICS OF SOME PALINURAN AND ASTACURAN CRUSTACEA[1]

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INTRODUCTION

Only a few serological tests indicating the taxonomic relationships among the long-tailed Crustacea are recorded in the literature. Nuttall (1904) and Graham-Smith (1904), both using the same antisera made against *Homarus vulgaris* and *Potamobius astacus,* separately tested these antisera with a large variety of animal sera; of these antigens only those of the decapod Crustacea gave positive reactions. The results obtained by these authors, in some instances, were not in accord with accepted zoological classifications. The serum of *Palinurus vulgaris,* for example, reacted more weakly with the anti-*Homarus vulgaris* serum than did the sera of certain brachyuran species. Erhardt (1929) produced an antiserum against the serum of the crayfish *Potamobius astacus* and tested it with the homologous serum, plus the sera of *Potamobius leptodactylus,* the lobster *Homarus vulgaris,* several brachyuran species, and other invertebrates. Except for an unexplained heterologous reaction with the serum of the snail, *Helix pomatia,* only the crayfishes and lobster reacted, and these in accordance with the taxonomic positions previously assigned to them on the basis of information obtained from non-serological data. That the sera used as antigens by these early workers were not standardized in terms of protein content may, in part, explain the unexpected results sometimes obtained when the reactivity of their apparently non-comparable antigen dilutions were compared.

Clark and Burnet (1942) using the ring test sensitivity method, and unstandardized antigen dilution series, were able to separate various genera of the Parastacidae from each other and from other Decapods.

In 1943, Boyden, using a turbidimetric analysis of the whole precipitin range, measured the degree of serological correspondence between the sera of *Homarus americanus* and *Homarus vulgaris* with an appropriate antiserum. The index of serological activity used by this author was a value proportionate to the whole curve area. This was the first application of a quantitative technique to obtain serological data that was to be used in judging the taxonomic relationships of the long-tailed Crustacea.

The present writer made a preliminary report (Leone, In press) of his investigations of the sera of invertebrates, and included in it some of the results reported below. The object of this present report is to record further serological data that will aid in making a more accurate taxonomic arrangement of the palinuran and astacuran Crustacea.

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MATERIALS AND METHODS

As a result of the establishment of the Serological Museum of Rutgers University (Boyden, 1948), cooperating institutions and individuals from several parts of the world have aided in collecting and providing the samples of animal sera used in this investigation. Sera of species of the Australian genera *Euastacus* and *Cherax* were obtained through the efforts of Miss Ellen Clark of Victoria. Sera of the Hawaiian species of *Panulirus* were provided by Dr. Bradley T. Scheer of the University of Hawaii. Sera of the American west-coast spiny lobster were provided by Dr. Albert Tyler, California Institute of Technology, Pasadena, California. The other samples of sera came from stocks in the Serological Museum, which were collected chiefly by Dr. Alan A. Boyden, Rutgers University. Table I contains a list of all the antigens used and the antisera prepared.

TABLE I

A list of the species, and their sources, whose sera were used in making the serological comparisons reported upon in this paper

Species	Sample	Source	Antiserum
Panulirus argus (Latreille)	39–1 ♂	Tortugas, Florida, U.S.A.	I–76
Panulirus argus (Latreille)	39–3 ♂	Tortugas, Florida, U.S.A.	I–77
Panulirus interruptus (Randall)	46–1	California Institute of Technology, California, U.S.A.	I–56
Panulirus penicillatus (Oliver)	49–1	Honolulu, Hawaii	I–117
Panulirus japonicus (de Siebold)	49–1	Honolulu, Hawaii	
Palinurus vulgaris Latreille	No. 1	Plymouth, England	I–93
Homarus vulgaris Milne-Edwards	No. 1	Plymouth, England	I–91
Homarus americanus Milne-Edwards	36–2d	Mt. Desert Island, Maine, U.S.A.	I–89
Cambarus clarkii Girard	36–1	(Unknown)	I–92
Euastacus elongatus Clark	48–1	Victoria, Australia	I–116
Euastacus nobilis (Dana)	48–1	Victoria, Australia	
Euastacus armatus (von Martens)	48–1	Victoria, Australia	
Cherax destructor Clark	48–1	Victoria, Australia	
Cherax albidus Clark	48–1	Victoria, Australia	

The details of the technique of testing have been considered elsewhere (Boyden, 1942; Boyden and DeFalco, 1943; Leone, 1949). Briefly, the method employed is to measure, by means of a photoelectric turbidimeter, the precipitate developed in an antigen dilution series, after a constant amount of antiserum has been added to each dilution. The range of antigen concentrations extends from complete antigen excess to complete antiserum excess. Also considered in these earlier papers were various problems related to establishing the validity of the results of tests made with sera collected over a period of years, and which were processed in various ways. In general, the serum antigens proved to be representative of the organisms from which they came, and were suitable reagents with which to conduct researches in serological systematics. The customary twenty-minute reaction time at 38° C. was employed in these tests. In examining the degree of serological correspondence [3] among the sera of the various species in the genera *Panulirus* and *Euastacus* a twenty-four hour reaction time at room temperature was employed, as well as the short time readings.

EXPERIMENTAL RESULTS

The results of the serological tests are summarized in Table II and Table III.

TABLE II

Serological Relationships

Antigens	Antisera							
	Panulirus argus	*Panulirus interruptus*	*Panulirus penicillatus*	*Palinurus vulgaris*	*Homarus americanus*	*Homarus vulgaris*	*Cambarus clarkii*	*Euastacus elongatus*
Panulirus argus	100	86	88	35	5	5	6	4
Panulirus interruptus	85	100	88	31	5	5	5	4
Panulirus penicillatus	88	88	100	33	4	4	4	4
Panulirus japonicus	70	71	77		13		4	
Palinurus vulgaris	54	31	50	100	4	7	5	3
Homarus americanus	14	9	15	5	100	96	50	10
Homarus vulgaris	15	11	16	6	88	100	48	10
Cambarus clarkii	13	11	14	6	50	48	100	38
Euastacus elongatus	13	11	14	6	54	49	57	100
Euastacus nobilis	13	10	14	7	53	49	58	89
Euastacus armatus	13	11	13	7	53	47	55	88
Cherax destructor	12	9	11	6	47	47	65	61
Cherax albidus	10	8	12	6	48	45	63	58

The numbers in the table are percentages of the value of the homologous reaction which is designated as 100 per cent. Values within 5 per cent of one another are generally considered to be within the experimental error of the method. All values are averaged from two or more tests, except those for *Panulirus japonicus*. It is readily seen that the various antisera differ in their capacity to react with the heterologous sera. All values are for 20 minute reaction time.

Of the species tested in the genus *Panulirus,* a high degree of correspondence is shown by the sera of the spiny lobsters, *P. argus, P. interruptus,* and *P. penicillatus.* On the basis of the serological evidence alone, these three species are in the same category, or are in closely related categories, whereas *P. japonicum* is in another category.[4] The European spiny lobster, *Palinurus vulgaris,* is in still a different category and the degree of difference between it and the others is so great as to suggest differences on the generic level.

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[3] Serological correspondence. As applied to antigens it includes all antigens capable of reacting with an antiserum to any one of them. As applied to antibodies, or antisera, it includes all antibodies or antisera capable of reacting with a given antigen or hapten. Whenever the serological correspondence is exact we may speak of serological equivalence (Boyden, 1942).

[4] The serum sample of this species had a soluble protein concentration of less than one per cent, and, in addition, was preserved in merthiolate colored with eosin. Consequently the contents of the tubes in the antigen excess region of the reaction curve were dark red. The amount of reaction recorded seemed to be less than a visual estimate of the dilution tubes would indicate. The antiserum excess region of the curve was normal. Any change, applied as a correction, would be in the direction of a closer correspondence to the homologous antigen indicating less difference between *P. japonicum* and the other palinuran species than is indicated in the table.

TABLE III

A comparison of 20 minute and 24 hour reaction times

Species	Reaction time					
	20′	24 hr.	20′	24 hr.	20′	24 hr.
Panulirus penicillatus	100	100	88	100	86	96
Panulirus argus	88	102	100	100	85	99
Panulirus interruptus	87	100	88	99	100	100
Euastacus elongatus	100	100				
Euastacus armatus	88	98				
Euastacus nobilis	89	96				

Values indicate the per cent relationship to the homologous reaction. The antigenic correspondence indicated after 20 minutes is high; after 24 hours correspondence in antigen reaction is exact.

The true lobsters, *Homarus americanus* and *Homarus vulgaris,* also show a high degree of serological correspondence. Whether or not the difference between them is sufficient to merit separate specific status for each is open to question from the serological point of view.

The three species of the genus *Euastacus* closely correspond to one another serologically, the heterologous reactions being only 11 to 12 per cent less than the homologous reaction. As it was in the cases of the palinuran organisms, these astacuran species show less than the 30–50 per cent difference from the homologous reaction which has been suggested (Boyden, 1943) as defining species in serological tests involving decapod Crustacea. As Boyden pointed out (personal communication), these values depend upon the grade of specificity of the antisera used and have reference to comparable antisera only.

The serum of *Cambarus clarkii* is notably different from the sera of the other astacuran species. The sera of species of the fresh water genera *Euastacus* and *Cherax* react more strongly with the anti-*Cambarus* serum than do the sera of the true lobsters of the genus *Homarus.* The sera of *Homarus,* on the other hand, react considerably more with anti-*Cambarus* serum than do the sera of any of the palinuran, spiny lobsters. Sera of species of the genus *Cherax* gave slightly greater reactions with the anti-*Euastacus* serum than did the serum of *Cambarus.*

Various species of brachyuran Crustacea were tested with each of the antisera shown in Table I. Heterologous reactions were obtained in some tests, indicating serological correspondence. In no test, however, did the serum reaction of any brachyuran exceed, or approach for that matter, the weakest heterologous reaction of the palinuran and astacuran species.

In order to ascertain whether the differences in the reactivity of heterologous antigens of high serological correspondence were constant, some of the values for twenty-minute reaction times were compared with twenty-four hour reaction times. A period of twenty-four hours at room temperatures was sufficient time for the precipitin reactions to go to completion. Merthiolate employed at a concentration of 1 : 10,000 in the saline diluent was adequate to inhibit bacterial growth during the test period.

From the data in Table III it can be seen that the heterologous test values which differ from the homologous by as much as fifteen per cent after twenty-minutes reaction time, may appear to be serologically equivalent after twenty-four hours of reaction time. Reactions with values within five per cent of the homologous value are considered equivalent. The sera of the three listed species of the genus *Panulirus* appear to be serologically equivalent, after twenty-four hours, as are the sera of the three species of the genus *Euastacus*. When the correspondence of the sera of heterologous species is as high as it was in these tests, experience has taught us that detectable differences, as revealed by other criteria, are slight. Consequently the usefulness of serum as a precise taxonomic tool is demonstrated. These observations are pertinent for the sera of Crustacea which possess only a single kind of antigenic protein, the hemocyanins (Allison and Cole, 1940; Clark and Burnet, 1942; Tyler and Scheer, 1945), and are, by their nature, relatively pure serological reagents. Serological reactions with these sera are not so subject to the inconsistencies and irregularities which sometimes are revealed, with proper techniques, in mixed antigens such as the sera of mammals which contain several antigenically distinct proteins (Serological Museum, Bulletin No. 2, 1948).

Summary

From the serological studies of the palinuran and astacuran Crustacea it is concluded that:

1. Sera of species within a genus react more strongly with an antiserum made against one of them than with any other antiserum.
2. Sera of different species within the same genus may show different degrees of relationship to an antiserum made against the serum of one member of that genus.
3. The sera of representatives of different genera in the same family show different degrees of relationship to one another.
4. The sera of species within a family react to a greater extent with antisera made against the sera of members of that family than with any other antisera.
5. *Panulirus argus, Panulirus interruptus,* and *Panulirus penicillatus* correspond almost exactly with one another serologically, and on the basis of the serological evidence presented, may be considered the same, or closely related species. This is in spite of their wide separation geographically.
6. *Euastacus elongatus, Euastacus nobilis,* and *Euastacus armatus,* serologically, closely correspond with one another. Probably they are the same, or closely related, species.
7. In the genus *Homarus,* the two species *H. americanus* and *H. vulgaris* closely correspond with each other, and probably are the same, or closely related species.
8. Differences in per cent of serological relationship among crustacean sera tested with the same antiserum are attributable to differences in their hemocyanins.
9. Twenty-minute reaction times for precipitin reactions establish significant orders of relationship among animal sera. Twenty-four hours reaction time does not alter the order of relationship from that established at twenty minutes despite considerable increases in amounts of reaction recorded.

10. Differences detectable among antigens of high correspondence, after a twenty-minute reaction time, may disappear after a reaction time of twenty-four hours.

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LYTIC EFFECTS OF SPERM EXTRACTS ON THE EGGS OF MYTILUS EDULIS[1]

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INTRODUCTION

A variety of specific substances are obtainable from the eggs and sperm of animals (reviewed by Tyler, 1948, and Bielig and von Medem, 1949). From the sperm may be obtained antifertilizins and various lytic substances such as hyaluronidase from mammalian sperm, an egg surface lysin from sea-urchin sperm, and membrane lysins from the sperm of various animals.

As described in a preliminary note (Berg, 1949) sperm extracts of *Mytilus edulis* are capable of dissolving or preventing the formation of the cement which normally binds the segmentation blastomeres together. The present report concerns the characteristics of the active agent particularly with respect to its chemical nature and relationship to an egg membrane lysin which is also present in the sperm extracts. The cement dissolving activity of the extracts is closely associated with the membrane lysin; however there is evidence which suggests that the effect is brought about by a separate substance.

Lysis of jelly coats and membranes of eggs by sperm extracts has been described by previous investigators. Hibbard (1928) and Wintrebert (1929, 1933) discovered that strong sperm suspensions or sperm extracts of the amphibian, *Discoglossus*, caused dissolution of an egg coat. Tyler (1939) extracted membrane lysins from the sperm of the keyhole limpet, *Megathura crenulata*, and the abalone, *Haliotis crackerodii*, which dissolved the membranes of the eggs. von Medem (1942, 1945) subsequently described similar membrane lysins in other species of limpets and abalones. Ruffo and Monroy (1947) reported the dissolution of the egg coats of several species of sea-urchins by a hyaluronidase-like substance obtainable from the sperm, and Monroy (1948) later reported the presence of a membrane lysin in the sperm of *Pomatoceros*.

The action of the above membrane lysins on cleavage of the eggs was in some instances not reported or claimed to have no effect.

METHODS

The gametes of the bay mussel, *Mytilus edulis*, were used for the majority of these experiments, although at times the testes of the larger species, *Mytilus califorianus*, were used for preparing considerable quantities of sperm extracts. Animals, collected from the San Francisco Bay or the open coast, were kept at 5° C.

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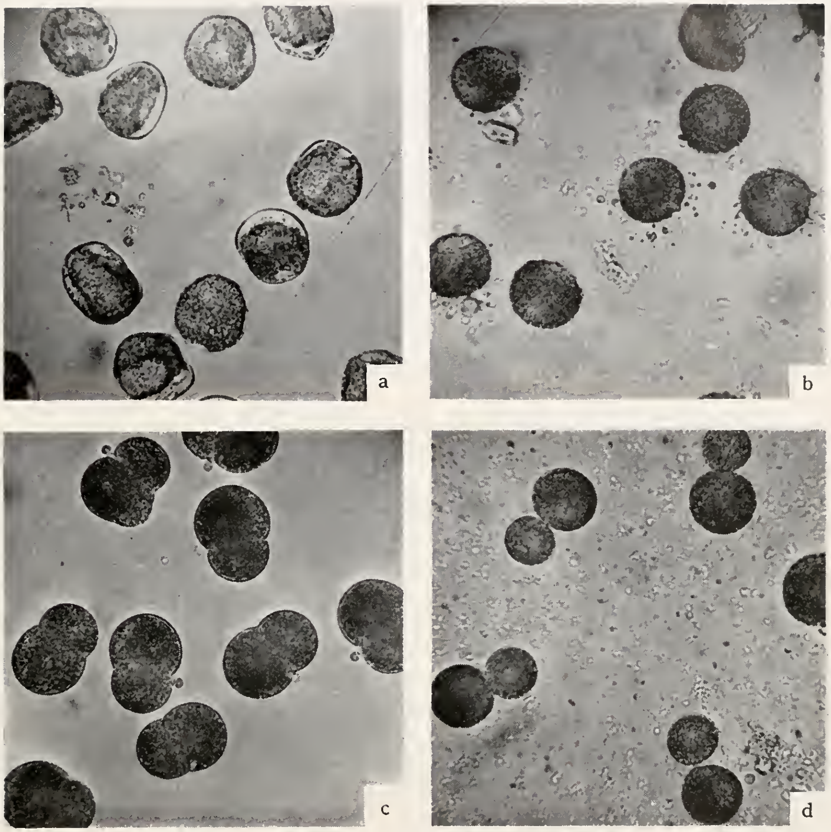
Plate 1



Effects of *Mytilus edulis* sperm extracts on the egg membrane and intercellular cement: a, membranes rendered visible by plasmolysis of eggs with hypertonic sea water; b, dissolution of membranes after two minutes treatment with a sperm extract; c, normal appearance of eggs after first cleavage; d, cleavage of eggs in a sperm extract.

for as long as three or four days. Viable eggs and sperm were obtained by allowing the animals to spawn in individual finger bowls of sea water at room temperature.

Sperm suspensions, used for the preparation of sperm extracts, were obtained either by concentrating naturally spawned sperm by centrifugation or by the shedding of sperm from a number of excised testes in a small volume of sea water. The latter procedure proved to be particularly convenient for obtaining very dense suspensions of sperm. Sperm extracts were prepared by freezing a sperm suspension followed by thawing and grinding the frozen mass in a mortar. This resulted in a viscous liquid which on centrifugation separated into a white precipitate and a straw colored supernatant. The latter, representing a crude sperm extract, contained the egg membrane lysin and cement dissolving factor.

Ripe eggs of *M. edulis*, as obtained by spawning, are about 63 microns in diameter, somewhat opaque, and irregularly shaped. There is no egg coat or jelly layer although a thin membrane can be made visible by cytolysis of the egg in distilled water or by plasmolysis in hypertonic sea water. The latter treatment was used as a standard method for revealing the membranes for subsequent tests in sperm extracts. Sea water concentrated to nearly twice the normal strength was used as a plasmolytic agent, and after 10–20 minutes exposure to this solution the membranes became clearly visible (Plate 1a).

The cement dissolving activity of a sperm extract was determined by whether the first cleavage blastomeres became noncohesive and spherical. Since the membranes themselves act as physical hindrances to separation or rounding up of the blastomeres, they were routinely removed from the eggs shortly after fertilization. As a standard procedure the membranes were removed by a strong sperm extract 10–20 minutes after fertilization of the eggs. The "naked" eggs were transferred through several dishes of sea water to remove all traces of the sperm extract and then added to the test sperm extracts 10–15 minutes before the first cleavage.

EFFECTS OF SPERM EXTRACT ON EGG MEMBRANES AND INTERCELLULAR CEMENT

The presence of an egg membrane lysin in *M. edulis* sperm extracts is easily demonstrated. An extract prepared by the method of freezing and thawing will cause the disintegration of the membranes in a few seconds or minutes depending upon its strength. The dissolution begins by a thinning and buckling of the membrane. Gradually the sharp edges become less distinct until only a residue remains as a corona about the egg surface. In all except weak sperm extracts, this corona eventually disappears. Plate 1a shows the appearance of the membranes after plasmolysis of the eggs and 1b shows eggs of the same sample two minutes after treatment with a sperm extract.

The preceding plasmolytic treatment necessary to expose the membranes has no effect on the lytic process since the membranes can be dissolved as readily from unplasmolyzed eggs.

Sperm extracts, in addition to dissolving the membranes, also have a striking effect on the early cleavages of the eggs. Cleavage of *M. edulis* eggs has been described and pictured in detail by Field (1922) and only a brief account need be given here. Shortly after fertilization the eggs lose their irregular shapes and by the time the first polar body is formed they are spherical. Shortly after the second polar body is formed the egg surface in the vegetal hemisphere becomes wrinkled, followed by a bulging of the cytoplasm in this area. The cytoplasmic bulge, or polar lobe, causes the egg to assume a pear shape (Fig. 1a) and as the lobe increases in size the egg becomes flattened and furrowed in the animal hemisphere (Fig. 1b). This gives the illusion that the egg has divided into three cells, the so-called trefoil stage. The first cleavage plane, beginning at the animal pole, curves to one side in the vegetal hemisphere, so that the polar lobe is separated from the AB cell, but retains connection with the CD cell (Fig. 1b). Fusion of the polar lobe with the CD cell occurs soon after the trefoil stage resulting finally in an unequal cleavage. The first cleavage blastomeres are strongly cohesive and there is a considerable flattening of their area of contact (Fig. 1c, Plate 1c).

If eggs are allowed to cleave in a sperm extract, the appearance of the polar lobe and blastomeres is quite different from the normal pattern. The polar lobe appears as a bulge in the vegetal hemisphere, as in the controls, and the eggs assume a pear shape (Fig. 1d). However, shortly afterwards, at what would correspond to the trefoil stage, the appearance diverges from that of the controls. At this stage (Fig. 1e) the polar lobe extends directly out from the eggs as a globule of cytoplasm connected to the CD cell by a stalk. The plane of cleavage is straight instead of curved and as the furrow advances across the cytoplasm the newly formed edges become separated instead of cohering as in the controls. After cleavage the polar lobe is retracted into the CD cell, although occasionally it may be pinched off completely. At the close of first cleavage, the blastomeres are spherical and may be either completely separated or barely touching (Fig. 1f, Plate 1d). In a weak sperm extract the blastomeres remain attached, but the area of contact is noticeably reduced as compared to the controls.

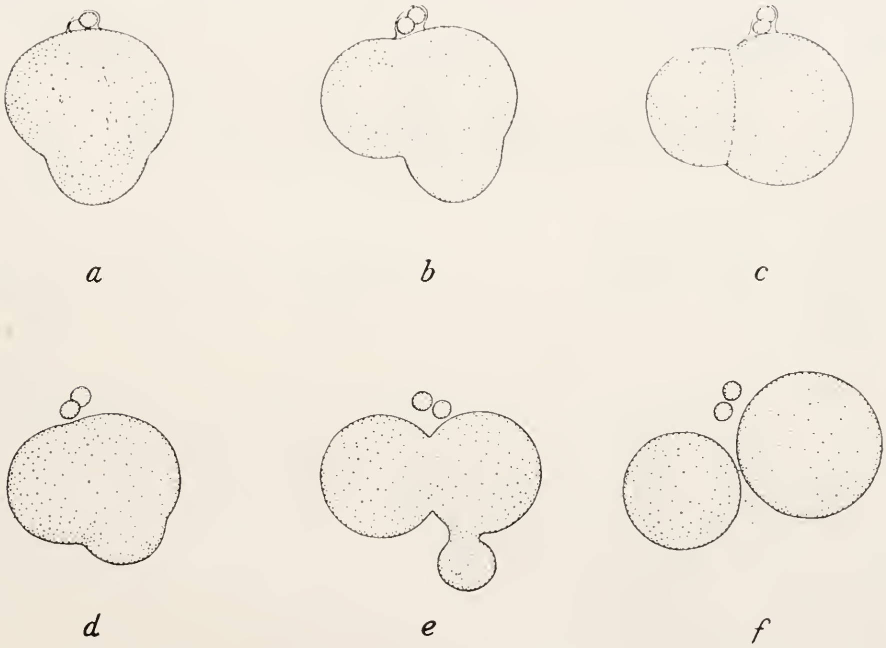


FIGURE 1. Normal first cleavage of *Mytilus edulis* eggs (a-c) and cleavage in a sperm extract (d-f) : a, formation of polar lobe ; b, trefoil stage ; c, completion of first cleavage ; d-f, corresponding stages of cleavage in a sperm extract.

The cement dissolving activity of sperm extracts is maximal during the process of cleavage. Eggs which have already completed the first cleavage are affected very little. However, if they are allowed to remain in the extract, the A, B, C and D cells, formed by the second cleavage, become noncoherent and assume a spherical shape. Embryos which are reared in sperm extracts usually develop into a loose mass of ciliated cells.

Essentially the same pattern of cleavage and development is obtained if the eggs are allowed to cleave in calcium free sea water. Although there are slight differences in appearance of eggs cleaving in a sperm extract as compared to those in calcium free sea water, it seems reasonable to assume that the sperm extract acts in a similar manner as lack of calcium—by dissolving or preventing the formation of the cement which holds the cells together.

Fertilized eggs which have had the membranes removed, often become sticky and adhere to the bottom of the containers. Addition of a sperm extract causes the adhesiveness to disappear which supports the view that the extract contains a substance capable of dissolving the cement. This, in the broad sense of the term, may be considered a lytic activity of the sperm extracts and the active agent will be referred to tentatively as the cement lysin.

PROPERTIES AND RELATIONSHIP OF ACTIVE AGENTS IN THE SPERM EXTRACTS

A lytic action of sperm extracts on the intercellular cement has not been described before, either in *Mytilus* or other forms, and it seemed worth-while to investigate the causative agent in regard to its occurrence, identity or non-identity with the membrane lysin, and some of its chemical properties.

TABLE 1

Bioassays of the lytic activities of various types of sperm extracts.
Each figure represents a separate experiment.

	Time for membrane dissolution of minutes	Titer of cement lysin
Concentrated sperm suspensions	4, 6	16, 32
Supernatants of above suspensions	Slight effect after 45 min. to 1 hour	4, 8, 8
Extraction by freezing and thawing	2, 5, 3	128, 64, 64
Extraction by heat	No effect	16, 16
Acid extraction	5, 5	32, 64
Alkaline extraction	15	16

In many of the experiments to be described, it was necessary to use bioassays of the sperm extracts. The time interval for dissolution of the egg membrane is a satisfactory assay for the membrane lysin; however, there is no such convenient time factor involved in the dissolution of the cement. Accordingly a dilution assay was employed in which the sperm extract was diluted ½, ¼, ⅛, etc., and the greatest dilution at which a perceptible effect occurred indicated the strength of the original sperm extract. The strength or titer of the extract was most conveniently expressed as the reciprocal of the greatest dilution exhibiting an effect. A strong sperm extract may have a cement dissolving titer as high as 128; accordingly there is a considerable range for comparison of different extracts. Using these bioassays, the strengths of the effective agents were determined in sperm suspensions, their supernatants, and in extracts prepared by various methods. Results of typical experiments are summarized in Table 1.

Dilute sperm suspensions (as obtained by spawning) and their supernatants had no perceptible effect on the egg membranes or early cleavages. Sperm suspensions, roughly about fifty times as concentrated as above, were prepared by the shedding of the sperm from excised testes. These suspensions dissolved the membranes about as rapidly as extracts prepared from the same suspensions, but the cement dissolving titers were proportionately weak as compared to the extracts (Table 1). This difference, however, seems too slight to be interpreted as any real separation of lytic activities. The supernatants, as obtained by centrifugation, caused dissolution of the intercellular cement, but had an extremely weak lytic effect on the membranes. This was originally interpreted as a separation of active agents; however, assays of the supernatants showed that the relative strengths were comparable to those normally found in an extract. Thus one-eighth dilutions of extracts prepared by freezing and thawing resulted in membrane-lytic strengths equivalent to that of the above supernatants and cement dissolving titers from 4 to 8 which are also equivalent to that of the supernatants.

Dissolution of the membranes and intercellular cement is brought about by sperm extracts prepared by acidification or alkalization. A portion of a concentrated sperm suspension was brought to pH 1.5 by addition of HCl, centrifuged and the supernatant neutralized. The supernatant had nearly the same activity (Table 1) as a comparable extract prepared by freezing and thawing. Considerably weaker extracts were obtained by alkalization (pH 10 or above); however, the relative strengths of the lytic activities were the same as by other methods of extraction.

Sperm suspensions after heating to 100° C. for a few minutes yielded supernatants which dissolved the intercellular cement, but lacked the membrane lysin; however, this was not a differential extraction, since, as will be pointed out later, the membrane lysin is destroyed by heat.

It became evident that the lytic activities of sperm extracts were very closely associated and could not be separated readily by these methods into two specific fractions. Accordingly, further tests were carried out to determine some of the chemical properties of the active agents in the hope that these tests might lead to conclusions regarding their relationship.

The active agents of the sperm extracts are evidently large molecules as shown by their non-dialyzability. In a typical experiment 50 cc. of sperm extract were dialyzed in a cellophane tube for two days in an equal volume of sea water. Tests of the dialysate on the egg membranes and early cleavages were negative, whereas the solution inside the tube retained nearly the original strength.

Precipitates obtained by 50 per cent to 90 per cent saturation with $(NH_4)_2SO_4$ or by dialysis against distilled water contain the active factors. Precipitation by $(NH_4)_2SO_4$ was brought about by slowly adding at 3° C. a saturated solution of the salt to the sperm extract until the desired concentration was reached. At 50 per cent saturation a floccular white precipitate usually formed within a few hours. Since centrifuging tended to disperse rather than to concentrate the precipitate, it was allowed to settle out for a period of 24 hours and the supernatant decanted. This did not give complete separation, but was sufficient to show that the bulk of the activity of the sperm extract was in the precipitate. Table 2 records assays of the lytic activities of the precipitate and supernatant. The residual activity of the supernatant was probably due to incomplete separation of the precipitate and perhaps also to incomplete precipitation of the active substances.

A heavy and complete precipitation of the active factors was obtained at 90 per cent saturation with $(NH_4)_2SO_4$ (Table 2). Concentrations intermediate between 50 per cent and 90 per cent gave results similar to a 50 per cent saturation. No differential precipitation of active agents occurred.

Dialysis against distilled water also brings about precipitation of the active substances from a sperm extract. In a typical experiment, 50 cc. of sperm extract were dialyzed against 4 liters of distilled water at 3° C. Dialysis was carried out for 48 hours although a precipitate began to appear within several hours. Assays

TABLE 2

Precipitation of lytic activities of a sperm extract

Treatment of sperm extract		Time for dissolution of egg membranes	Titer of cement lysin
50% saturation with $(NH_4)_2SO_4$	supernatant	15 minutes	4
	precipitate	1/2 minutes	64
90% saturation with $(NH_4)_2SO_4$	supernatant	No activity	No activity
	precipitate	2 minutes	Very strong (titer not determined)
Dialyzed against distilled water	supernatant	15 minutes	4
	precipitate	1 minute	32
Precipitation by adding alcohol		No activity	8
Control sperm extract (prepared by freezing and thawing)		1 minute	64

of these showed high lytic activities of the precipitate as compared to the supernatant (Table 2).

Since a differential precipitation of the lytic factors did not occur, it cannot be concluded on the basis of the above results that there are two different lysins present in the sperm extracts. The presence of two active agents is, however, suggested by a differential effect of heat on the sperm extracts. Table 3 records the results of typical heat tests on a *M. edulis* sperm extract. Samples of the extract were heated at various temperatures for five minute periods and then assayed for the lytic activities. Five minutes at 60° C. was sufficient to inactivate partially the membrane lysin, but had little effect on the cement lysin. Nearly complete inactivation of the membrane lysin occurred within 5 minutes at 80° C.; however, sperm extracts after one hour at 100° C. still caused dissolution of the intercellular cement, but showed no membrane lysis.

TABLE 3

Differential inactivation of lytic activities of a sperm extract by heat

Treatment of sperm extract	Time for dissolution of egg membranes	Titer of cement lysin
5 minutes at 60° C.	5 minutes	64
5 minutes at 80° C.	20 minutes	32
5 minutes at 100° C.	No activity	16 to 32
1 hour at 100° C.	No activity	8
Control	2 minutes	64

The heat stability of the cement dissolving activity of sperm extracts depends in part on the pH of the extract. At pH 3 to pH 8 there is little difference in the rate of inactivation by heat; however, at pH 10 the cement lysin is completely inactivated within a few minutes at 100° C.

Alcoholic precipitation of sperm extracts causes complete inactivation of the membrane lysin and partial reduction of the cement dissolving activity. Chilled ethyl alcohol was slowly added to a sperm extract at 2° C. until a final concentration of 80 per cent was reached. A white precipitate formed which was freed of alcohol and dissolved in sea water. This solution exhibited no membrane-lytic activity; however, its cement dissolving titer was 4 to 8 as compared to 64 for the original extract.

The lytic activities of sperm extracts are destroyed by trypsin. In a typical experiment a trypsin solution was added to a strong sperm extract resulting in a final concentration of 0.1 per cent of the enzyme. After several hours treatment of the extract with trypsin, the extract had no discernible effect on the egg membrane or intercellular cement, indicating that the active agents had been destroyed.

Extracts, prepared by freezing and grinding, of various tissues (other than the testes) of adult mussels exhibited no lytic activities on the eggs.

Sperm extracts may be dried under vacuum without loss of activity, a procedure which has proven useful for obtaining concentrated solutions of the active factors. Concentration of sperm extracts by this method or by precipitation in salt free solutions led to the discovery of an inhibitor of the cement lysin. Although only a low percentage of eggs cleaved in a concentrated extract, the blastomeres of eggs that did cleave were nearly as cohesive as the controls. Assays of the extract showed a high titer of the cement lysin, but its effect was not noticeable until about a $\frac{1}{4}$ to $\frac{1}{8}$ dilution of the original extract. Apparently there was an inhibitor in the extract which over a threshold concentration prevented the dissolution of the cement. Little is known about the nature of this inhibitor except that it does not inhibit the membrane lysin, and that it is destroyed in a few seconds at 100° C.

SPECIES SPECIFICITY

Tyler (1939) reported that the membrane lysin from *Megathura* sperm does not dissolve *Haliotis* egg membranes and von Medem (1942, 1945) concluded on the basis of more extensive tests that the membrane lysins of various mollusks are to some extent species specific.

Active sperm extracts are obtainable from both *M. edulis* and *M. californianus* and cross tests were carried out to determine the specificity of the lytic activities. *M. californianus* extracts were able to dissolve the membranes and intercellular cement of *M. edulis* eggs; however, the reverse tests were negative. An *M. edulis* extract had a very weak lytic effect on the *M. californianus* egg membrane and no discernible effect on the intercellular cement.

A few cross tests were carried out on the eggs of other animals. *M. californianus* and *M. edulis* membrane lysins did not dissolve egg membranes of *Acmaea scabra, Mya arenaria, Urechis caupo,* and *Strongylocentrotus purpuratus* nor did sperm extracts of these animals have any effect on their respective egg membranes or the *M. edulis* egg membrane. With the exception of a few experiments, only the membrane-lytic action of the sperm extracts was tested since the membranes of most of the eggs could not be removed for conclusive tests of possible cement lysins. *M. californianus* sperm extracts, however, had no effect on the hyaline layer of sea-urchin eggs after removal of the fertilization membranes.

DISCUSSION

For the purpose of comparison, the main characteristics of the lytic activities of *M. edulis* sperm extracts are summarized in Table 4. It is evident from inspection of this table that the characteristics of these agents closely parallel one another. Similarities exist not only in occurrence and methods of extraction, but also in the chemical behavior. There is no conclusive proof that there are two active agents present in the sperm extracts although this possibility is strongly suggested by a differential inactivation of the activities by heat. The membrane lysin is extremely heat labile, being partially inactivated in a few minutes at 60° C. and completely inactivated in a short time at higher temperatures. In comparison the cement dissolving activities is relatively heat stable and is only partially inactivated after one hour at 100° C. This greater stability of the cement lysin is also reflected by retention of activity after precipitation in alcohol, whereas the membrane lysin is completely destroyed by this treatment.

TABLE 4

Characteristics of lytic activities of M. edulis sperm extracts

Membrane lysin	Cement lysin
1. Maximum activity obtained by acidification or freezing of sperm suspensions	1. Maximum activity obtained by acidification or freezing of sperm suspensions
2. Not obtainable from other tissues.	2. Not obtainable from other tissues
3. Non-dialyzable	3. Non-dialyzable
4. Inactivated by trypsin	4. Inactivated by trypsin
5. Heat labile	5. Relatively heat stable
6. Precipitated by dialysis against distilled water	6. Precipitated by dialysis against distilled water
7. Inactivated by precipitation in alcohol	7. Partially inactivated by precipitation in alcohol
8. Precipitated in 50% to 90% solutions of $(NH_4)_2SO_4$	8. Precipitated in 50% to 90% solutions of $(NH_4)_2SO_4$
9. Not inhibited in a concentrated sperm extract	9. Inhibited in a concentrated sperm extract

Precipitation by alcohol, $(NH_4)_2SO_4$, and salt free solutions strongly suggests that the active substances are proteins, which, by definition, would be globulins. Inactivation of both the membrane lysin and the cement lysin by trypsin also suggests their protein nature. The enzymatic nature and probable protein structure of the membrane lysin of *Megathura* sperm has been previously pointed out by Tyler (1939) and evidently the membrane lysin of *Mytilus* is similar in nature.

The mechanism of action of the cement lysin is not known. Although it is only slowly inactivated by heat an enzymatic action cannot be completely ruled out. It may act in a way so as to prevent coupling of calcium with the cement protein; however, it is unlikely that this is brought about by binding of free calcium in the sea water. Excess calcium in a sperm extract does not decrease the effectiveness of the extract in causing dissolution of the cement. Also if the mechanism were a binding of free calcium one would not expect a species specificity of sperm extracts. That a species specificity does exist is shown by the fact that *M. edulis* extracts have no effect on *M. californianus* eggs and furthermore they do not dissolve the hyaline layer of membraneless sea-urchin eggs.

Fauré-Fremiet and Thaureaux (1949) have described in detail the action of a number of synthetic detergents on the eggs of *Toredo norvegia.* Separation of the first blastomeres of *Toredo* occurs in weak solutions of detergents in much the same way as a sperm extract separates those of *Mytilus.* Supposedly, detergents are able to interact with and disperse proteins and it may be that the cement dissolving action of a *Mytilus* sperm extract has a similar mechanism of action.

It is unlikely that there is any relation between the cement dissolving activity of *Mytilus* sperm extracts and hyaluronidase of mammalian sperm. The latter is enzymatic in action and acts on the hyaluronate substance of mammalian tissue (reviewed by Meyer, 1947), but has no effect on the intercellular cement of blood capillaries (Chambers and Zweifach, 1947). Also the hyaluronidase-like substance of sea-urchin sperm (Ruffo and Monroy, 1947) has no effect on the intercellular cement of the sea-urchin embryo. It might also be pointed out that beef testis hyaluronidase has no perceptible effect on the membranes and cement of *M. edulis* eggs.

Runnström, et al. (1945, 1946), described a lytic activity of methanol extracts of sea-urchin sperm which apparently caused liquefaction of the egg surface as indicated, in part, by constriction of eggs into fragments at high centrifugal forces. The active agent is heat stable, dialyzable through cellophane, and is not inactivated by trypsin. A few preliminary tests of the cement lysin of *Mytilus* indicated that it did not alter the irregular shapes of unfertilized eggs or facilitate constriction of eggs during severe centrifugation. Thus the egg-surface lysin of sea-urchin sperm and the cement lysin of *Mytilus* show considerable differences in their chemical nature and effects on the eggs.

The function of the several lysins obtainable from the sperm of animals is not clear although possible roles in fertilization have been suggested. A reasonable explanation for the presence of the membrane lysin, as suggested by Tyler (1939), is to aid penetration of the sperm through the egg membrane to the surface of the egg. Although the agent causing dissolution of the intercellular cement may occur in physiological concentrations, it cannot be concluded without further work that it has a role in fertilization.

SUMMARY

Extracts of *Mytilus* sperm contain a lytic substance, or substances, which causes dissolution of the egg membrane and the intercellular cement which binds the blastomeres together. Bioassays were utilized to investigate the occurrence and relationship of the active agents and some of their chemical properties. The lytic effects are exhibited by concentrated sperm suspensions and their supernatants obtained by centrifugation. Extracts prepared by freezing and thawing and by acidification or alkalization exhibit the same relative strengths of lytic activities.

The active agents are large molecules, as indicated by their non-dialyzability through cellophane. They are precipitated by ammonium sulfate, by dialysis against distilled water, and by alcohol. These properties indicate a protein nature of the lytic substances, an assumption which is further supported by the fact that they are inactivated by trypsin.

While there is no conclusive evidence that there are two separately acting lysins, this is strongly suggested, in part, by a differential inactivation of sperm activities by heat. The membrane lysin is heat labile whereas the cement-lytic activity is relatively heat stable. It is concluded that the latter effect is not brought about by a binding of free calcium of the sea water.

The author is indebted to Dr. M. Schlamowitz for advice on biochemical methods and to Dr. R. M. Eakin for helpful comments and criticisms of the manuscript.

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CARTESIAN DIVER RESPIROMETER

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In this paper is described a modification of Linderstrøm-Lang's Cartesian diver method (1937) specifically adapted for the measurement of slight variations in the respiration of cellular objects. The diver is a further development of the diver used by Zeuthen (1946) for measuring respiratory rhythms in the egg of the frog.

Essential Features of the Method

The diver (Fig. 1) has a gas volume of only about 1 μl. It is kept floating at a constant level throughout the experiment. Therefore no disturbing currents are set up in the flotation medium by the diver itself and the saturation equilibria within the diver (see below) are not influenced by such more or less sudden changes in pressure in the gas bubble as would result if the diver was allowed to go to the bottom of the flotation vessel between the measurements. The gas volume in the diver is confined to one air bubble only. Therefore only one meniscus separates the gas space in the diver from the surrounding medium. All these factors are considered essential for obtaining accurate respiration measurements. One more factor is of paramount importance: since the diver is supposed to float in a medium which does not itself flow, it is necessary to avoid heat convection currents in the flotation vessel. Such currents will result from temperature variations in the thermostat. Not only the magnitude of possible temperature variations is important, but also the rate of variation should be kept low. A very fine thermostat, operating with as gentle heating and cooling as possible, is necessary. If not, the temperature regulation itself, usually operating on the principle of alternating heating and cooling, will result in convection currents in the flotation vessel. Therefore, in most cases it is much more convenient to work in a well-stirred water bath which is not regulated at all, but which adjusts itself to the room temperature. If the water bath is large and the room temperature fluctuates only slightly, the temperature will change very slowly. In this way no disturbing convection currents are initiated. The total change in temperature of the bath can be kept within very moderate limits if the room temperature is relatively constant. The change in pressure in the diver is automatically adjusted for temperature changes because the other side of the manometer is connected to the enclosed gas volume in a large bottle (2 liters).

The diver consists of a diver chamber, bottom droplet with animals, gas bubble, and alkaline, CO_2-absorbing neck fluid. The gas bubble forms a complete separation between bottom and neck fluids (this point is discussed at some length by Giese and Zeuthen (1949)). A hollow glass stopper is inserted in the neck of the diver. The stopper serves several different purposes: it retards diffusion of gases to or from the surroundings, gives the diver buoyancy and permits the pressure-sensitive air volume of the diver to be reduced almost ad libitum. Since the glass stopper is inserted without any grease, narrow passages are left which allow the air bubble in the diver to react with volume changes to outside pressure variations, created by the aid of an adjustable water manometer. The diver floats in the same medium which is in the neck of the diver; e.g., in alkaline water, isotonic with the biological medium in the bottom droplet. A long glass extension may be attached to the outer end of the stopper. This makes the diver float upside down ("inverted diver"), so that the organisms in the diver, if immobile, will come to rest on the meniscus between air and water, not on the bottom of the diver, away from the air bubble.

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[1] Fellow of the Rockefeller Foundation.

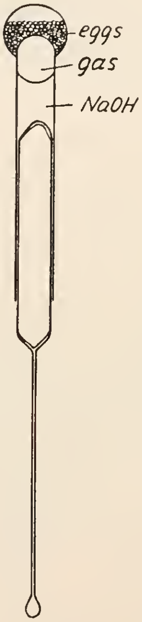

FIGURE 1. The diver.

Experiments with the diver are reported in the paper by Zeuthen (1950b). Figure 2 in that paper indicates that the diver is practically tight to gases. However, in the first part of the experiment, an apparent respiration is often measured. This is explained by assuming that a diffusion equilibrium is gradually becoming established inside the diver. If, for instance, the diver is filled with fluids which are in equilibrium with air, but an oxygen bubble is introduced, gas goes in solution because oxygen is more soluble in water than is air: Equilibrium is established asymptotically. How long it takes before it is well enough established depends mainly on the gases involved, on the relative amounts of water and gas in the system, and on the diffusion distances in the water of the diver. In the diver used for the experiments on eggs of *Urechis,* relatively much water was present in the system. Therefore the initial equilibration period was long (1–2 hours). It is important to remember that for disclosing a possible rhythm in respiration, the initial period need not be over. If it is not over, the rhythmic respiration is merely superimposed on a smooth curve which asymptotically approaches the X-axis.

During the respiration experiment, the pressure in the system decreases. At the same time as oxygen is used from the bubble, gas which is dissolved in the water in the diver again goes out of solution, back to the bubble. The condition of equilibrium spoken of becomes a quasi-equilibrium in which the oxygen tension in the water and in the bubble drops at the same rate. Thus oxygen is taken from water and from bubble in proportion to the amounts of oxygen present in both places. In the author's experiments on *Urechis,* approximately 6 to 10 per cent of the oxygen was present in the water, and the total respiration is measured too low by this value. For the measurement of variations in oxygen uptake this means practically nothing (comp. Linderstrøm-Lang, 1946). However, as soon as absolute respiration measurements are to be performed, these conditions have to be taken into consideration by applying corrections or by reducing the ratio between water and gas in the diver.

Any variation in respiratory rate must show up somewhat delayed because a diffusion path is introduced between the respiring objects and the air bubble. In the inverted diver (with objects on the meniscus) this is usually not a significant factor, however. In the experiment on *Urechis* (diffusion distance less than 0.5 mm) the delay was probably less than 1–2 minutes. Moreover, the fluctuations in respiration were recorded without any significant damping. These statements are theoretical deductions based on Linderstrøm-Lang's calculations (1946). However, the experiments of Blinks and Odenheimer (personal information), who used this diver in studies on photosynthesis in single algal cells, seem to verify these deductions. If the dimensions of the diver are changed from those shown in Figure 1, Linderstrøm-Lang's paper (1946) on diffusion systems in micro respirometers should be consulted.

Making, Calibrating and Filling the Diver

The diver is made from a thin-walled capillary tube with a diameter conveniently a little less than 1 mm. The ratio between inner and outer diameter should be close to 0.9. Such capillaries may be drawn from thin-walled test tubes which should be heated by rotation in a broad flame. From the thin-walled capillary two pieces are selected, of which one fits tightly into the other. The former piece is used for the stopper, the latter for the diver chamber. Principles of glass blowing which are given by Holter (1943) are employed. For the micro-burner a hypodermic needle is very convenient. With rich natural gas (as in California), the oblique tip of the needle serves to mix the gas and air. It is best to work with a very small flame (3–4 mm long) and it is often useful to mount the micro burner on the table of a dissection microscope. It is desirable to keep the volume of the sealed stopper small (3–4 times the volume of the "pressure sensitive" gas bubble) in order to make the diver as sensitive to pressure changes as possible.

Before use the diver should be tested and calibrated. For this purpose the diver chamber is filled completely with water and the stopper is inserted below water. Since the cross section of the capillary used for making the diver is usually not quite circular, the stopper will only slip into the diver chamber if it is correctly oriented. It requires some practice to find this orientation, and it may be useful to mark both tubes conveniently. This, however, has not been done by the author because very often useful reference marks (small dots, easily recognizable curvatures and the like) can be found on the diver. The diver thus filled should sink in its normal flotation medium. If so, small air bubbles are introduced into the diver chamber until the diver floats. For the introducing of air bubbles the diver is taken out of the medium and the stopper is gently pulled back, without completely removing it, however. Small air bubbles will then enter the diver along the stopper. If the gas volume of the floating diver is too large or too small, glass is removed from or added to the tail of the stopper. The many small air bubbles in the diver are allowed to unite into one. Then, below water, the stopper is pulled out and the air bubble is taken into a calibrated Holter braking pipette (Holter, 1943). The amount of air to be introduced into this diver before each experiment is indicated by the length of the air column in the braking pipette used.

A great advantage of the present method is that the diver can be charged freehand. Before the experiment the diver is rinsed by sucking first sulfuric acid and then water through the diver chamber by means of an ordinary pipette which is introduced into the submerged diver chamber. The measured volume of air is taken up into the braking pipette, and after this the biological object(s). In the filled pipette, therefore, we have in the lower end the respiring cells, and in the upper end the measured air volume. The pipette is gently blown out into the diver chamber which has previously been completely filled with water. If done under the dissection microscope it is fairly easy to deposit a large number of cells in the bulb of the diver and to place the air bubble where desired in the capillary, thus trapping the cells. After the experiment the cells may be removed from the diver and counted. It has (rather unexpectedly) been found that a complete and safe separation between bottom droplet and neck seal is formed if the air bubble is big enough to make a circular but very short (about 0.2–0.3 mm.) zone of adherence to the glass wall. Creeping of fluid along the glass from neck to bottom has only been observed when dust particles happened to form visible bridges across the clean glass surface. After the neck fluid has been replaced with isotonic alkali which is run in with a pipette (care being taken not to touch the air bubble), the stopper is introduced and the diver is transferred by pipette into the flotation vessel. The flotation vessel, manometer, microscope, and thermostat, have been described by Holter (1943); for thermostat, however, see above.

Measuring the Equilibrium Pressure

When the respiration of an object is to be measured over short periods the equilibrium pressure as well as the time must be measured very accurately. The following procedure of "turning point determination" has given good results: the diver floats at about the same level all the time. Just before a reading is to be taken, the manometer is adjusted at such a constant level that the diver rises very slowly. However, due to the respiration processes, the diver becomes gradually heavier, so that it rises more and more slowly, comes to a standstill, and finally starts sinking. It is possible to make the diver turn at exactly the same level (± a few 0.01 mm.) in all measurements. When the diver turns, a stopwatch is started. The watch is stopped again when the next reading is due, and at the same time another stopwatch is started for measuring the next period, etc. In this way the pressure remains unchanged from just before to just after the diver turns, and the two branches of the manometer can therefore be read (to the nearest 0.1 mm.) immediately after the watches have been pressed. The equilibrium pressure measured is valid for the moment when the diver turns and this moment can be observed with an accuracy of some few seconds. The described method of measuring equilibrium pressures works best when the equilibrium pressure changes fast.

Sensitivity of Method

The accuracy of the method was studied in connection with the experiments on dividing *Urechis* eggs. With a diver having an air volume of 1 μl. the oxygen consumption measured in 5-minute periods was about $2.5–5 \times 10^{-3}$ μl. (change in equilibrium pressure 2.5–5 cm./5 min.). This respiration could be measured with an error of probably well below 1 per cent. Thus the sensitivity of the method is about $2.5–5 \cdot 10^{-5}$ μl.

Summary

A Cartesian diver method which is specially adapted for following variations in respiratory rate of small objects is described. Accurate respiration measurements can be obtained every few minutes. The diver is calibrated empirically and it is filled free-hand.

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RESPIRATION DURING CELL DIVISION IN THE EGG OF THE SEA URCHIN PSAMMECHINUS MILIARIS

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Introduction

In a previous paper (Zeuthen, 1946) it was reported that during early development of the egg of the frog (*Rana plathyrrhina*) each cell division is accompanied by a slight increase in oxygen uptake. The curve describing oxygen uptake as a function of time roughly approximates a sine curve. The division furrow of the first, second, and third cleavages always appears during a period of increase in oxygen uptake.

The experiments were performed in modified Cartesian divers. The rate of decrease in pressure in an oxygen bubble situated close to the egg was measured. The respiratory carbon dioxide, however, was not chemically absorbed, but held in solution by the egg and by a considerable volume of water bathing the egg. The diver system, of course, was not shaken, so that all exchange of gases had to take place over certain diffusion distances.

Both factors mentioned, *viz.:* the incomplete separation of O_2 and of CO_2 in the diver and the diffusion distances present in the system, make it somewhat doubtful how to evaluate the results. Linderstrøm-Lang (1946) made elaborate calculations on the results obtained by Zeuthen and concluded that the observed waves are probably slightly delayed and somewhat damped as compared with the curves which would describe the true respiratory processes in the egg itself. According to Linderstrøm-Lang the wave amplitude found (about 5 per cent of the respiratory rate in the minima between the waves) would probably indicate "true waves in the egg" with a maximum amplitude of 7–11 per cent of the respiration in the minima.

These experiments do not quite conform with those of Brachet (1934, 1935) working with the Fenn-respirometer. He found two considerably larger variations in respiratory rate per mitosis in the egg of *Rana fusca.* The discrepancy between the present author's experiments and those of Brachet remains unexplained. Brachet has expressed the hope of being able to repeat his experiments (1945).

In a recent letter in *Nature,* Tang (1948) claims many years ago (Tang, 1937) to have found slight mitotic variations in oxygen uptake in the sea urchin egg, however only at a high temperature (25° C.). Tang considers that the failure of Gray (1924) to find any change in oxygen uptake in the dividing sea urchin egg is due to the fact that the experiments were performed at a low temperature (11° C.). Tang as well as Gray used the Warburg technique. Unfortunately, Tang has not been able to supply a copy of the original paper which was published in a Chinese journal. All records were destroyed during the war. Also Runnström (1934) found slight fluctuations in respiration in dividing sea urchin eggs. According to this author there is a maximum of respiration during prophase.

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None of the experiments mentioned, however, have appeared convincing to the reviewers in the field. The problem of respiration during mitosis is not solved yet. Linderstrøm-Lang's calculations on Zeuthen's experiments on the frog egg indicated that in future experiments with the Cartesian diver we would have to employ more intensely respiring eggs than the frog's egg, and we would have to secure complete carbon dioxide absorption in the diver. Both conditions are fulfilled in diver experiments on eggs of the sea urchin *Psammechinus miliaris* to be reported in this paper (a short note on this work has already been published in *Nature,* 1947). The curve found for this egg must therefore be considered rather accurately to describe the true processes in the egg itself. The experiments on *Psammechinus* are concentrated on later divisions (nos. 4–8), and the main object of the investigation is to establish possible mitotic variations in the respiration of this egg. The experiments were carried out in July–Aug., 1946. In a following paper on the egg of *Urechis* the first three or four divisions are considered, and on this object the exact time relations between respiration and stage of mitosis are given.

Material and Methods

Eggs of *Psammechinus miliaris* (Z-form) were fertilized artificially. It was not always possible to obtain batches of eggs which divided 100 per cent and synchronously, but if those eggs which were the first to enter the first division were picked out with a braking pipette and kept separately, these eggs invariably were found to divide well (95–100 per cent) and synchronously (i.e., within 5 min.) through a series of successive divisions. For each experiment 300–400 eggs were isolated in this way, the jelly was removed with a pipette, and about 200 eggs were transferred to the diver; the rest were kept as controls outside the diver in the same thermostat. In the diver the eggs were densely packed, but they were very close to a bubble of oxygen. The oxygen supply was calculated to be ample. Usually the first 5–6 divisions could be observed in the diver itself (not on all eggs, but on a representative number of eggs). In some cases, however, and especially for later divisions, I depended on observations of the controls in determining the cleavage times in the divers. In earlier stages, where comparisons could be made between division rates in-and-outside the divers, the rate of development was found to be uninfluenced by the stay in the diver.

The way of securing egg material which divided synchronously restricted this investigation to the later stages of development. The divisions nos. 4–8 were studied, these divisions taking the egg from the 8-cell to the 256-cell stage.

Two types of divers are used—neither of them very different from the one previously described (Zeuthen, 1950a). Both float with the stopper upwards, however, so that the eggs lie on the bottom of the diver, separated from the air space by a thin layer of water. The distance from the most remote eggs to the air space did not exceed 0.7 mm. in any experiment. The diver which is shown in Figure 1-A is the most sensitive because it contains a very small oxygen bubble (about 0.2 μl.). This air bubble is not large enough to separate the neck fluid from the bottom fluid.

The fluid which fills the neck and bathes the eggs is a CO_2 binding buffer: glycylglycine m/10 or m/50 in sea water, with pH adjusted to 8.2 by the addition of NaOH (pK of the amino group in glycylglycine being 8.07 according to Schmidt (1938, p. 613)). The buffer solution is made isotonic with sea water. During the division periods with which we are concerned (Fig. 2), the m/50 buffer was not observed to influence the development of the eggs at all, but in the m/10 buffer a slight retardation of the later cleavages (7–8) was noticed, and the blastulae did not swell quite normally. In the diver the oxygen bubble was kept down, close to the eggs, by the aid of a tiny glass extension on the stopper. The diver shown has the exact dimensions of the diver used in the experiment reproduced in Figure 2.

The diver shown in Figure 1-B is almost identical with the one used for the experiments with *Urechis,* the main difference being that it floats with the stopper upwards. Figure 1-B gives the exact dimensions of the diver which was used in

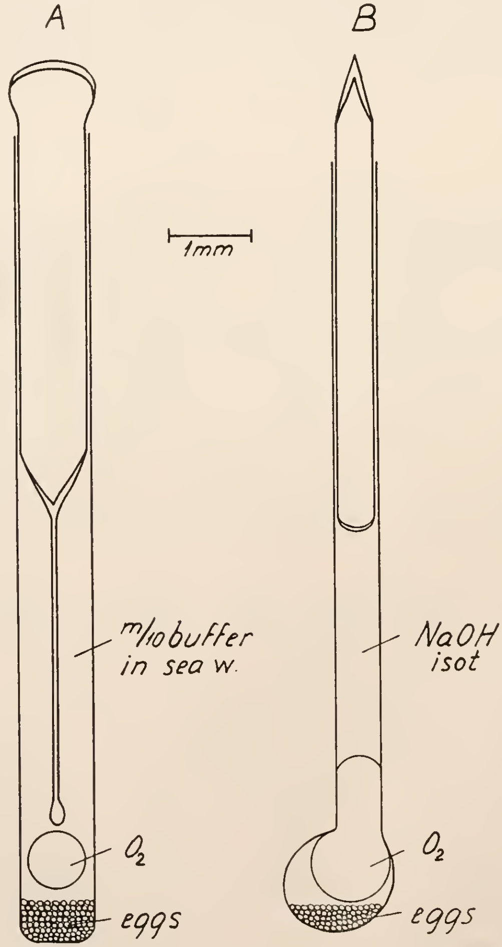

FIGURE 1. The divers used.

all three experiments of Figure 3. The air space (0.93 μl.) separates the bottom fluid with the eggs from the isotonic NaOH neck fluid. The diver floats in isotonic NaOH. Only the later cleavages (7–8) and the hatching period in *Psammechinus* have been studied with this diver. The accuracy of these experiments is not so high as it is now obtained in experiments with *Urechis*. Probably the temperature control in the thermostat (running sea water) could have been better.

Both types of divers were filled with pure oxygen. During the first part of the experiment, O_2 is used for saturating the fluids inside the diver. As mentioned elsewhere (Zeuthen, 1950a, c), this tends to make the measurements too high in the first part of the experiment. A possible respiratory rhythm should manifest itself even during this early period, however.

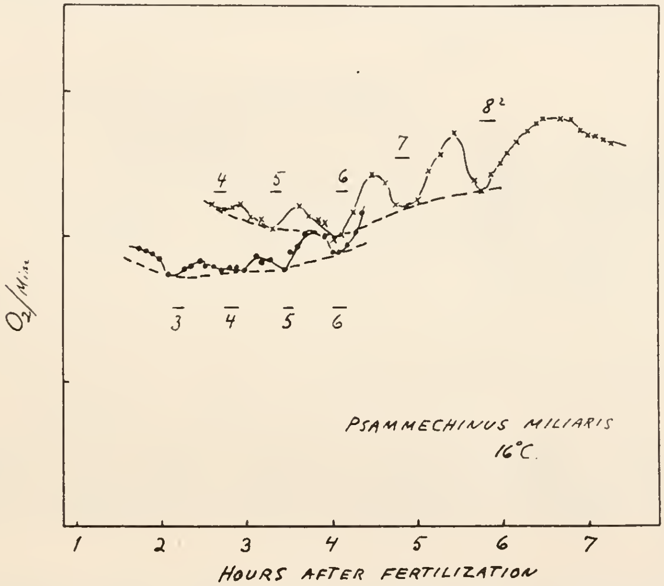

FIGURE 2. Two experiments with the diver shown in Figure 1-A, buffer m/10 glycylglycine.

The CO_2 absorption in both groups of experiments is considered complete. Since, moreover, the distance between the most remote eggs and the air bubble is small in all experiments, the damping of the respiratory waves found may amount to 5 per cent only, and the delay with which they are measured to less than two minutes. This can be determined from the tables in the paper of Linderstrøm-Lang (1946). Both the damping and the delay mentioned are insignificant, considering the not too high accuracy with which the dimensions of the respiratory waves can be recorded.

The temperature of the bath was regulated at about 16° by rapidly running sea water. The equilibrium pressure was determined as described by Zeuthen, 1946, 1950a (turning point determinations). The apparatus used was built in a very simple way at the Zoological Station, as far as possible following the directions of Holter (1943).

EXPERIMENTS

Figure 2 shows two experiments with the diver of Figure 1-A. The ordinate gives the respiratory rate in terms of change (in cm. Brodie) per minute of the equilibrium pressure of the diver. The abscissa gives the time in hours after fertilization at 16° C., the 5th cleavage occurring 3 hours, 20 minutes after fertilization.[2] Each measurement is plotted in the middle of the period for which it is valid.

After the initial period with too high readings is over, the true slope of the two curves is indicated. The main trend of the curves seems to conform fairly well with the S-shaped curve of Lindahl (1936) showing the increase of respiration of the sea urchin egg during the cleavage period. However, waves are superimposed on this curve, one wave per mitosis. The amplitude of the waves increases as development proceeds and as more and more simultaneous divisions occur in the embryos. The cytoplasm cleaves during the period of decreasing respiration, in some cases perhaps even when the respiration is at a minimum.

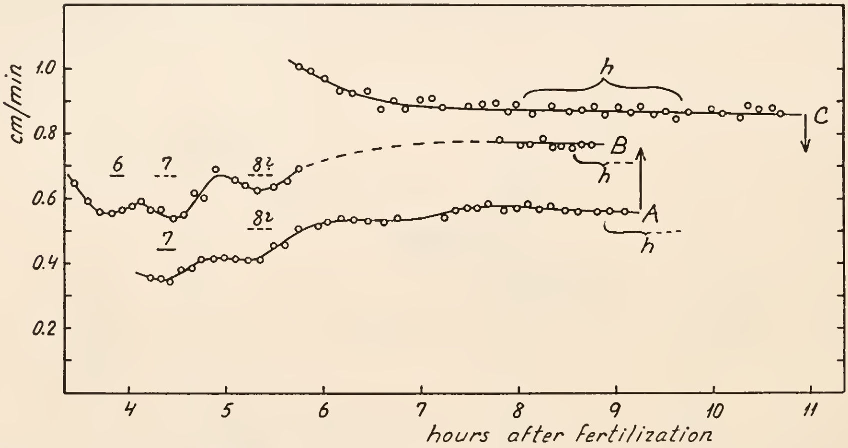

FIGURE 3. Three experiments with the diver shown in Figure 1-B. Actually all three curves should have been plotted at the same level as curve B. For the sake of clearness the curves have been separated in space. The arrows indicate the displacement. *h* indicates the hatching period.

All experiments of Figure 3 were performed with the diver shown in Figure 1-B. These experiments differ from those of Figure 2 in that the eggs lay in natural sea water throughtout the experiment, CO_2 being chemically absorbed by the alkaline neck fluid. The curves of this figure form a continuation of those of Figure 2. The eggs were introduced into the divers at the 64-cell stage or even later, and in one experiment (C) the respiration was followed until hatching of all eggs had taken place in the diver. Saturation equilibria are not established until about 1 hour after start of experiment. Cleavages 6 to 8 seem to be accompanied by large variations in respiratory rate; later the respiratory variations again become slower and slighter, and for a long time after, no variations whatsoever in respiratory activity were disclosed, despite the fact that all the larvae hatched one by one during this period.

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[2] Dr. Hans Borei, Stockholm, has pointed out to me that the time relations on my curve in *Nature* are not quite correct (Borei, 1948). In *Psammechinus* the 5th cleavage (at 16° C.) appears 3 hours, 20 min. after fertilization, not after 4 hours, 30 min., as indicated in my figure. I have not myself measured the time (at 16° C.) from fertilization to start of the single experiment, because the necessary manipulations during this period involved serious temperature changes. From this and other reasons the said curve was only intended to give a rough survey to indicate for the reader on which part of the respiratory curve in the sea urchin the respiratory waves are located. However, I thank Dr. Borei very much for his information which I have taken advantage of in timing the curves of Figures 2 and 3 of this paper in relation to the time of fertilization.

The general trend of Figure 3: a continued rise in metabolic rate followed by a period of constant respiration during the period of hatching conforms again with Lindahl's S-shaped curve—in this case with the later half of the S.

Discussion

The experiments indicate rather conclusively that the mitoses in the sea urchin egg are accompanied by changes in respiratory rate. But the interpretation remains uncertain in this respect: Is the increase or the decrease in respiration the important thing? As a matter of convenience, we shall in this paper interpret the curves as indicating that each mitosis is accompanied by a slight excess oxygen uptake. The respiration increases at some early stage of the mitosis, attains a maximum and decreases again at the end of the mitosis when the cytoplasm cleaves.

A smooth auxiliary line, which just touches all minima of the experimentally found curve, will separate a number of small areas above the auxiliary line from a large area below this line. According to the accepted point of view the large area below the line would indicate some kind of "basal metabolism" of the eggs, whereas the small areas would measure the "excess metabolism" of the individual mitoses.

The upper curve of Figure 2 shall now be analyzed in detail in order that we can get some idea of the relative magnitude of the "basal" and of the "excess" metabolism of the division cycle numbers 5–8 incl. in the egg of *Psammechinus*. There is a difficulty here because the measured respiration is known to be too high in the initial period of the experiment (see above). In this particular experiment, however, the diver had been in the flotation vessel for about ¾ of an hour before measurements were taken. Calculation by the formulae of Linderstrøm-Lang (1946) indicate that after about another 1.5 hour the initial period of this particular diver (dimensions fig. 1-A) is practically over. The curve found seems to agree well with this view. After the 6th division the general trend of the curve conforms well with the curve of Lindahl. In the upper curve of Figure 2 we may therefore introduce a reasonable correction for the measured respiration in the first part of the experiment by extrapolating the curve found after the 6th division back to the time of 5th and 6th division. In this way we find that the respiration was approximately 25 per cent too high during the 5th division and about 10 per cent too high during the 6th division. This way of correcting is admittedly rather arbitrary, but in view of the comparatively low accuracy of the whole analysis, it is probably satisfactory.

Table 1 gives the results of the analysis.

TABLE 1

Division number	Number of cells dividing per embryo	Wave area	
		Absolute	Percentage
5	16	1.0	3.4
6	32	2.1	5.0
7	64	3.9	7.7
8	128	5.7	9.6

The different areas between curve and auxiliary line touching all the valleys are determined by weighing the areas after cutting the graph paper. In Table 1, column 3, the extra O_2 (absolute measure, independent on correction) taken up during the 5th division is taken as unity and it appears that during the 8th division approximately 6 times as much extra oxygen is consumed as that taken up during the 5th division. As indicated in column 4 the "excess oxygen consumption" is but a small fraction of the basal oxygen consumption in the same period, the figures being 3.4 per cent for the 5th division and 3 times as much (9.6 per cent) for the 8th division. The reason why the percentage extra O_2 increases less than 3-fold at the same time as the absolute extra O_2 increases 6 times is partly due to the fact that the "basal" metabolic rate increases with development, but mostly because also the time occupied by one division increases. It is unknown whether this longer division time in later stages is due to an extension of one or more stages of the mitosis proper, or to the appearance of an interphase. Although only one experiment has been considered good enough to permit a close analysis, qualitatively this experiment is supported by the other experiments published. The two experiments of Figure 2 are in good agreement in indicating the approximate size of the respiratory waves accompanying divisions 4–6. Also, the important experiments of Figure 3 show that the large waves accompanying the later divisions can be found when no artificial buffer systems are added to the sea water. In these experiments the waves were found in the initial period, e.g. before saturation equilibria had become established in the diver. For this reason we shall not try to make a quantitative comparison with the experiments of Figure 2.

The safest and at the same time the most promising conclusion to be drawn on the basis of Table 1 is that for the three successive divisions 5–6–7 there is a doubling of the "excess oxygen consumption" for each new division. Thus, over this period the extra oxygen taken up per mitotic cycle is approximately proportional to the number of cells dividing. This finding is very different from what was found for the first three divisions in *Urechis* (Zeuthen, 1950c) and in the frog (Zeuthen, 1946). In these two eggs the extra oxygen taken up per mitotic cycle is essentially constant despite the fact that the number of dividing cells increases from 1 to 4. In the early divisions the *Psammechinus* egg (comp. the lower curve in Fig. 2) seems to line up with two other eggs mentioned, so probably it is safe to conclude that in dividing eggs some physiological difference exists between the early and the later mitoses.

Table 1 seems to indicate that after the 7th division in the *Psammechinus* egg matters change again. The extra oxygen consumption of the 8th division is only 46 per cent higher than that of the 7th division. Figure 3, curve B, however, shows an 8th respiratory wave which is approximately twice the preceding one. In this experiment no buffer was added. Both figures (Figs. 2–3) agree in indicating a big 9th wave which is very extended in time, though. After this no more respiratory fluctuations could be detected although ciliation and hatching of the embryos took place in the diver. It is well known that the mitotic rhythm characteristic for the dividing egg does not continue forever. The data of Table 1 and the curves of Figures 2 and 3 are perhaps best explained by the assumption that at some time—presumably around the 7th–9th division—the mitoses in the *Psammechinus* egg gradually become significantly asynchronous, less numerous and perhaps restricted to specific areas of the embryo. We are gradually approaching the period of differentiation with which this paper is not concerned.

For the deep interest in the problems involved and for valuable help during the performance of the work at Kristinebergs Zoologiska Station the author is very much indebted to Professor John Runnström, University of Stockholm.

SUMMARY

The later divisions in the egg of *Psammechinus miliaris* are accompanied by variations in oxygen uptake. As in the embryo more and more cells divide simultaneously, the respiratory variations become more and more pronounced. The phenomenon is interpreted as an extra O_2-uptake correlated with the mitosis. During the four divisions nos. 5–8 proportionality is approximated between number of cells dividing and extra O_2 taken up. Thus, for the division period considered, the extra O_2 consumed by a dividing cell approximates constancy, *viz.:* it is independent of the size of the dividing blastomere.

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RESPIRATION AND CELL DIVISION IN THE EGG OF URECHIS CAUPO

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In a preceding paper (Zeuthen, 1950b) respiratory variations have been shown to accompany the later divisions in the egg of the sea urchin *Psammechinus miliaris*. In the frog egg the first three divisions are accompanied by faint, but clear-cut variations in oxygen uptake (Zeuthen, 1946). However, special conditions pertaining to large eggs in general (cf. the discussion in this paper) make it necessary to perform studies on a smaller egg similar to those on the frog egg. The present paper deals with the respiration of dividing eggs of the echiurid worm *Urechis caupo*. It is attempted to obtain accurate information about the shape of the respiration curve and to establish the time relations between respiration and stage of mitosis. Moreover, the recent communication by Tang (1948), claiming that in the sea urchin mitotic variations in respiration can be found at high but not at low temperature, has induced the present author to perform experiments on *Urechis* at widely different temperatures.

MATERIAL

The eggs of *Urechis caupo* have proved to be excellent objects for embryological and cell-physiological studies. From male and from female animals uncontaminated sperm and eggs can be drawn throughout most of the year and each animal furnishes good sexual products over a long period. The descriptive embryology of *Urechis* has been studied extensively by Newby (1940). After the addition of sperm the eggs undergo reduction divisions. These are followed by regular mitotic cell divisions, one division every 45 minutes at 15° C. Fertilization is usually almost 100 per cent and the divisions are synchronous within a few minutes in the same lot. After the 16 cell stage has been reached, the spiral cleavage of the egg becomes apparent, i.e., within the single embryo different quarters of blastomeres divide at different rates. The present study, therefore, is confined to the period in which the reduction divisions and the first 4 regular mitotic divisions take place.

METHODS

The Cartesian diver method as described by Zeuthen (1950a) was used. The diver had an air volume of 0.8 μl–1.1 μl. The water volume was 2–3 times this. Thus in the experiments with O_2 in the diver 90–94 per cent of the oxygen present was in the gas phase of the diver; hence 90–94 per cent of the oxygen consumed by the eggs was manometrically measurable.

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[1] Fellow of the Rockefeller Foundation.

After fertilization, 10 minutes were allowed to pass in order that it could be ascertained that the eggs were fertilized (to nearly 100 per cent). Then the diver was charged and transferred to the flotation vessel. After this about one hour elapsed before saturation equilibrium was established in the diver and reliable measurements could be obtained. Usually the mitotic divisions had not started until after this period, so that the 3 to 4 first mitoses could be studied.

The appearance of the cleavage furrows could be observed in the diver itself through the horizontal measuring microscope. Not every egg in the diver could be observed, but a representative number could always be followed. After the experiment the eggs were removed from the diver and brought under a regular microscope. The development was always as good as in the control outside the diver. In the diver the eggs lay densely packed in a layer the thickness of which, however, never exceeded 0.5 mm. Except in some few experiments at low temperature, about 400–700 eggs were employed in each experiment. As will appear below, the oxygen supply in all experiments was ample.

In the graphs the respiratory rate (expressed in cm. Brodie/min.) is plotted against the time. Each measurement is plotted in the middle of the period for which it is valid. The lines above the individual curve indicate the division periods.

Experiments

Diver without eggs, with unfertilized eggs, and with fertilized eggs

If a diver is charged in the usual way, but without respiring objects in the water, we obtain curves of the types I or II in Figure 1. In experiment I the diver contains a bubble of atmospheric air, in experiment II it contains pure oxygen. In the first part of the experiment we measure an apparent respiration, which is faint in experiment I, but very pronounced in experiment II. In both cases the apparent respiration can be explained by assuming that a saturation equilibrium is becoming established in the diver. If the diver is charged with an air bubble, there is a good chance that the fluids of the diver from the beginning are in equilibrium with the gas phase. The fact, however, that the diver will usually have become a little heated by the hand during the filling procedure may explain why, even in this case, we often measure a slight uptake of gas during the first part of the experiment (decrease in solubility of gases with increasing temperature). In experiment II (O_2 in diver) the fluids of the diver were from the beginning in an approximate equilibrium with atmospheric air. In contact with oxygen, gas is taken up because oxygen is more soluble in water than is air.

Curve III shows the results with about 700 unfertilized eggs in the diver (O_2 in diver). After the initial period of equilibration within the diver is over, we measure a respiration which is gradually increasing, the increase, however, following a very smooth curve without any indications of a rhythm. Evidently, the accuracy with which the respiration over short periods can be measured is very high, the largest deviations from the average being about 1 per cent and the error on the single measurement being very much smaller.

Curve IV shows an experiment with about 500 fertilized eggs in the diver (O_2 in diver). All eggs divided almost synchronously, and simultaneously with the eggs outside the diver. The lines numbered 1–3 above the curve indicate the division periods as they could be observed in the diver itself. The time interval indicated by each line shows the whole division period. The bulk of the eggs, of course, divided within a distinctly narrower time interval. The respiration of the eggs fluctuates in waves: there is one respiratory wave per cell division and the cleavage period of each mitosis appears just after the respiration has passed the maximum.

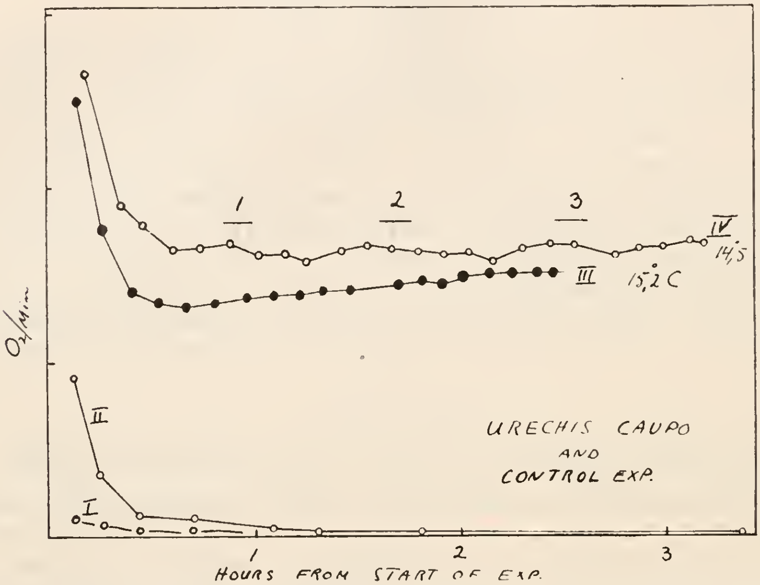

FIGURE 1. Control experiments with empty diver (I, II), experiment on unfertilized eggs (III) and on fertilized eggs (IV). 1st, 2nd, 3rd cleavages are indicated with horizontal lines.

A comparison between curves III and IV leaves little doubt that the respiratory waves are significant. Moreover, they have been found in all, e.g., about 10 experiments.

Experiments with dividing eggs at different temperatures. Oxygen in the diver

Tang (1948) considers the size of the respiratory waves accompanying cell division in the sea urchin egg to be highly influenced by temperature. The eggs of *Urechis* divide at temperatures ranging from about 9° C. to about 24° C. Above and below these temperatures cleavage becomes abnormal or fails altogether so that syncytia may develop. Experiments were carried out with normally developing eggs at 9.8–10° C., at 13–15° C. and at 22.8° C. Some of these experiments are assembled in Figure 2. The units on the ordinates have in each case been chosen so that the curves are directly comparable. The lower the temperature, the slower is the development and the less intense the respiration. Therefore, it is technically difficult to demonstrate the respiratory rhythm at low temperatures. The waves, however, are present at all temperatures. We shall return to this point in the discussion.

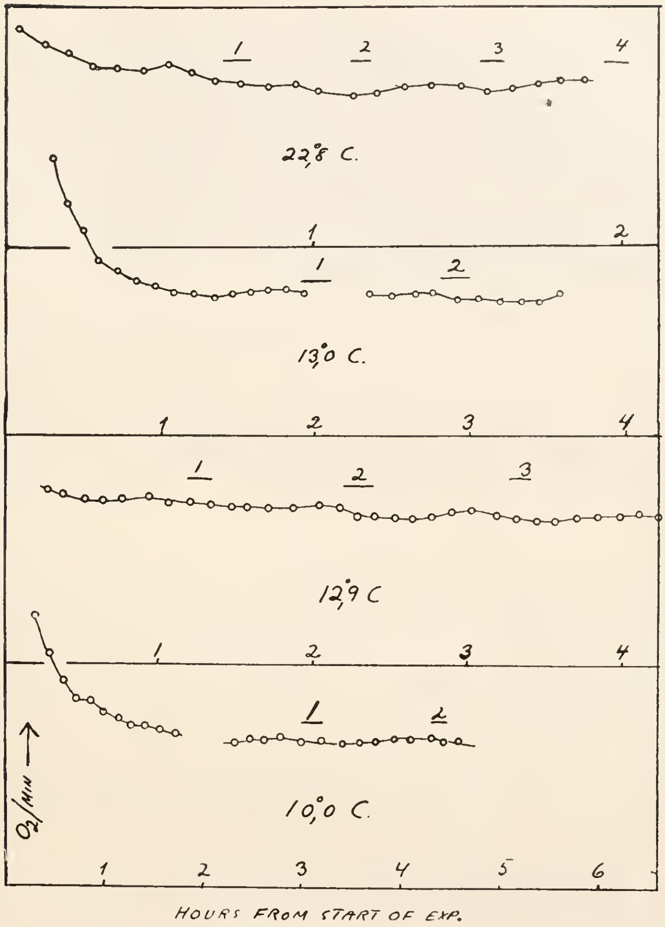

FIGURE 2. Within wide limits the respiratory waves appear to be uninfluenced by temperature.

Dividing eggs in a diver containing atmospheric air

The eggs seem to develop just as well in the diver where a high oxygen tension is prevailing as they do in the control (an open Petri-dish floating in the same thermostat as is used for the diver experiments). However, the respiratory waves might conceivably be influenced by the high oxygen tension in the diver without the cell division itself being in any way affected. The experiment, the result of which is shown in Figure 3, however, would seem to rule this possibility out. In this case the diver is filled with atmospheric air, and the eggs respire and divide normally until the 4th cleavage is completed. After this time signs of oxygen deficiency appear: respiration goes down and the cells divide more slowly than the controls outside the diver. The oxygen tension (expressed in per cent of one atm.) can, with fair accuracy, be calculated from the change in equilibrium pressure of the diver, assuming the oxygen pressure to be 20.9 per cent of an atm. at the moment when the diver is closed.

Curve II indicates oxygen pressure in the air bubble calculated in this way. The critical value seems to be about 6 to 7 per cent. This of course corresponds to even lower oxygen pressures on the respiring cells. The pressure difference between the gas phase and water which will allow enough oxygen to diffuse to the respiratory centers in this particular diver is evidently small. In most cases the divers which contained an oxygen bubble had about the same dimensions as had the diver used in the experiment of Figure 3. This would indicate that, in the oxygen experiments, the oxygen pressure in the eggs themselves was very close to one atmosphere. Thus, mitotic waves in oxygen consumption of dividing *Urechis* eggs have been demonstrated within wide limits of oxygen pressure.

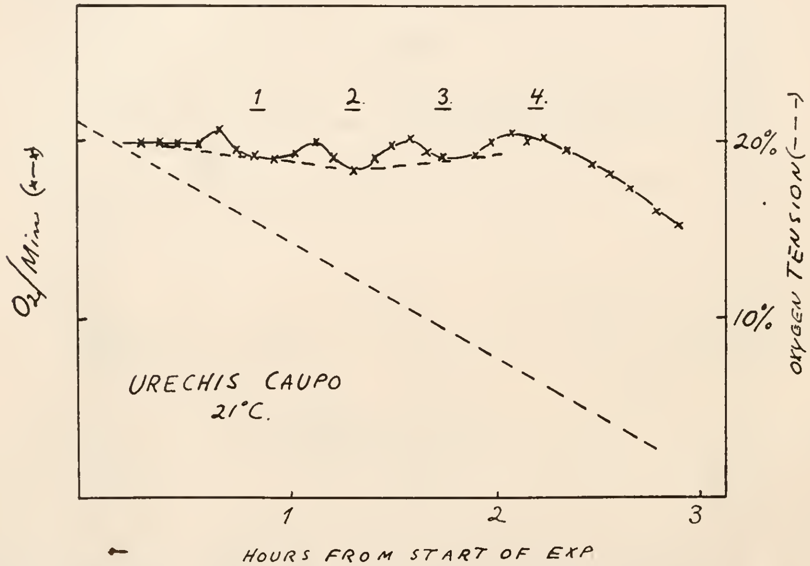

FIGURE 3. The respiratory waves can be demonstrated also at low oxygen pressures.

It may be worth while here to point out that the apparent difference between the curves of Figure 1 and Figure 2 is due only to a slight difference in the way of plotting. In Figure 1 the time scale is relatively long, and the respiratory waves therefore relatively long and flat. In Figure 2 the time scale is short and the waves shorter and steeper.

Oxygen uptake during the reduction divisions

There are some indications that the reduction divisions also result in slight changes in respiratory rate in the *Urechis* egg. In some cases the experiment was started so early that the reduction divisions occurred in the diver, though during the early part of the experiment at a time when the saturation equilibria were not yet established. However, two small waves seem to be superimposed on the curve, one wave per reduction division. Admittedly, these waves (to be seen on Fig. 2, 13°–10.0° C.) are very uncertain. Probably the safest conclusion to be drawn from these experiments is that, if the reduction divisions are accompanied by changes in the respiratory metabolisms, these changes are only slight.

Cytological observations

The time relations between stage of mitosis, cytoplasmic cleavage and fluctuations in respiration intensity were established in special experiments. Eggs from the same fertilization batch were kept in the diver and outside the diver at the same temperature and air or oxygen was bubbled through the outside suspension. Samples were drawn at regular intervals for cytological examination (Feulgen staining). The respiration was found to be low or increasing when the nuclei are in prophase. In those stages of actual mitosis when the nuclear membrane is dissolved away (meta-, ana-, and early telophase) the respiration is slightly increased. Maximum of respiration would seem to occur around metaphase-anaphase. Cytoplasmic division takes place during telophase at a time when respiration is already decreasing.[2]

The experiments indicate that the time relations between cytoplasmic cleavage and the different stages of mitosis are the same whether oxygen or atmospheric air is blown through the egg suspension. It should also be recalled that in all previous experiments the eggs cleaved at the same time in the diver (with air or with oxygen) as in the control (with air). All observations, tied together, support the general concept outlined above.

DISCUSSION

The respiratory rhythm found in dividing *Urechis* eggs very much resembles that previously described for the frog egg (Zeuthen, 1946). Due to a series of complicating conditions, however, Linderstrøm-Lang (1946) considered that the observed respiratory waves in the frog egg, while qualitatively true, were slightly delayed and somewhat damped as compared with the events in the cells themselves. The chief difficulty was that CO_2 was not chemically absorbed but only held in solution by a large volume of water. Moreover, the diffusion distances between the air bubble and the metabolic centers in the large egg were too long.

In the present experiments advantage has been taken of Linderstrøm-Lang's calculations. In the inverted diver the respiratory CO_2 is chemically absorbed and the eggs lie on the water-air meniscus, so that the distance between the gas bubble and the most remote eggs never exceeds 0.5 mm. (exception: experiment at 9.8–10° C., see below). The damping factor, φ, of Linderstrøm-Lang is between 0.99 and 1.00, meaning that the damping of the respiratory waves is less than 1 per cent. The delay, t_D, is about 1 minute. Thus damping and delay are well within the experimental error. Changes in respiratory rate that are not too fast are picked up and recorded accurately with the instrument used.

The situation is slightly different with the experiment at 9.8–10° C. Because of the low respiration more than 1000 eggs had to be used. Therefore, the layer of eggs plus water separating the most remote eggs from the gas phase was 0.85–1.00 mm. The damping of the amplitude of the respiratory waves may in these experiments have amounted to 10 per cent, and the delay in recording the waves may have been 3–4 minutes. In view of the fact, however, that the waves are very long at this temperature, the delay is insignificant. In Table 1, a 10 per cent correction for the lower wave amplitude in these particular experiments has been applied. A comparison of the curves in Figure 2 reveals that temperature can hardly be considered significantly to influence the respiratory waves in the *Urechis* egg. If Tang (1948), however, considers that the respiratory waves are technically more difficult to demonstrate at a lower than at a higher temperature, we agree completely.

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[2] The author wishes to record his sincere thanks to Dr. Irving A. Tittler, who made the microscopic preparations and studied the slides.

In Table 1 an intercomparison of all published curves is given. The wave heights are defined as described for the frog (Zeuthen, 1946). No certain dependency between the height of the respiratory waves and temperature or oxygen pressure can be observed. Figure 3 furnishes very slight evidence that the waves accompanying the later divisions may be slightly larger than those accompanying the first divisions. Figures 1 and 2, however, do not support such a view. The extra oxygen consumption of the first 3–4 divisions constitutes a very small fraction of the total respiration, e.g., 2–3 per cent only. Thus, the extra oxygen consumed per mitotic cycle is low, and probably it increases only slightly or not at all as we go from the 1st to the 3rd or 4th division. In this respect there seems to be a difference between the first divisions in *Urechis* and the later divisions (no. 5–8) in *Psammechinus*. It remains to be seen whether this is a difference between eggs or a difference between division stages, although the latter possibility appears to be the more likely one (Zeuthen, 1950b).

TABLE I

	Wave heights in % of total					
	in oxygen					in air
Temperature / Division no.	9.8°	10.0°	12.9°	13.0°	22.8°	21.0°
1	5.0	3.5	3.5	5.0	6.0	5.5
2	—	3.5	6.0	5.0	4.0	6.0
3	—	—	6.5	—	5.0	8.0

The physiological and biochemical function of the "extra oxygen" consumed during mitosis is unknown. However, since "mitotic variations" in oxygen uptake were found in a fertilized frog egg, which for some reason did not divide (Zeuthen, 1946), no close connection is indicated between respiratory processes and cytoplasmic cleavage. The fact that in *Urechis* (and *Psammechinus*) the cytoplasm always divides during a short period of decreasing respiration is an argument against the theories forwarded by some authors (discussed by Loeb, 1913) that the general increase in respiration which takes place while the sea urchin egg develops into a blastula correlates with the cytoplasmic cleavages and the resulting surface enlargement of the embryo. Moreover, comparing the eggs of the frog, the sea urchin and *Urechis* we find that the time relations between respiratory waves and cytoplasmic cleavage vary: In the two small eggs the cytoplasm cleaves in 2 or 3 minutes during telophase (decreasing respiration), but in the frog egg (*Rana fusca,* Brachet, 1934, 1935) the cytoplasm already starts to divide at the animal pole of the egg during prophase (increasing respiration). However, in the frog it takes a long time before the furrow embraces the whole egg. It seems probable that in all three eggs the time relations between stage of nuclear division and of respiratory changes may be much the same as found in the egg of *Urechis*. Thus the bulk of evidence is in favor of the theory that cleavage is more or less dissociable from the two more closely correlated processes of nuclear division and fluctuations in oxygen uptake. The experiments of Runnström (1934) to be discussed below throw some light on this latter correlation.

According to Runnström (1934) the first part of the mitosis in the sea urchin egg is strictly dependent on the presence of oxygen. In an atmosphere of nitrogen or in the presence of KCN the eggs are not capable of carrying out those steps of the mitosis which lead to the dissolution of the nuclear membrane. If, however, the eggs are brought under anaerobic conditions or if they are poisoned with KCN at any stage after the anaphase (diaster), the eggs will divide synchronously with the controls in air. Unfortunately, such experiments have not been performed on *Urechis*. Provided both eggs are really comparable, it would seem that oxygen is required during the period of increasing respiration, but that it is not necessary when the respiration is again decreasing. Perhaps, therefore, an increase in respiration in the first part of the mitosis is *indispensable* for the mitotic process in the sea urchin and in *Urechis*. In the frog this is *not* so. Brachet (1935) found that this egg is capable of development to the young blastula stage in the complete absence of oxygen. Here fermentative energy evidently can take the place of oxidative energy even during the first part of the mitosis.

Zeuthen (1946) mentions the fact that in the frog egg the mitotic variations in O_2-uptake can be interpreted not only as variations in the rate of oxidations in the egg. Even if the egg respires at an absolutely uniform rate the rate of O_2-uptake from the surroundings will vary with possible changes in the permeability of the egg surface to O_2. In the latter case the "respiratory waves" measured will actually only be variations in the amount of oxygen physically dissolved in the egg. The presented accumulation of further experimental facts on other, more intensely respiring and probably more permeable eggs, however, makes a uniform interpretation on the basis of the permeability theory of all results almost impossible. There seems to be little doubt, then, that what we measure is in reality variations in the rate of oxygen utilization in the eggs.

My sincere thanks are due to Dr. L. R. Blinks and to Dr. C. B. van Niel, both of Hopkins Marine Station, for the interest they showed in the present work.

SUMMARY

The first 3–4 mitotic divisions in the egg of the echiurid worm *Urechis caupo* were found to be accompanied by slight (2–3 per cent) increases in respiration. The phenomenon was found at all temperatures at which normal development takes place (9–24° C.), and it was found with air as well as with oxygen in the diver. The respiration was low or beginning to rise during prophase. It was maximum around metaphase-anaphase, lower during telophase when the cytoplasm divides.

In the discussion the experiments are considered in relation to previously recorded observations.

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COELOMIC PRESSURES IN SIPUNCULUS NUDUS

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Most invertebrate animals are characterized by lack of constancy of their internal pressure. In sipunculids, for example, the pressure may fluctuate within a short time between nearly zero and the equivalent of the blood-pressure of man. Pressure variations of this magnitude never occur within closed vascular systems. They are possible only in continuous spaces filled with fluid and enclosed by a muscular body wall. In such systems the main function of pressure is not circulation, but hydraulic work. In "closed" systems with more constant fluid pressure, hydraulic work is accessory. This latter function has been recently investigated in the annelid *Arenicola* by Chapman and Newell (1947). In the present study, pressure variations in *Phascolosoma* and *Sipunculus* will be analyzed mainly in relation to body movements. Coelomic pressure has long been recognized as essential in the digging activities of sipunculids; Uexküll (1903) measured in *Sipunculus nudus* pressures up to a maximum of 6 cm. Hg, i.e. 80.4 cm. of water, during burrowing in sand. Information on sipunculids is offered in the monograph of Baltzer (1934).

The hydraulic function of pressure in body fluids is wide-spread throughout the animal kingdom as shown in the following examples of water vascular systems. In holothurians pressure in the water vascular system is essential for tentacle erection (Zuckerkandl, 1948). In those sipunculids which possess tentacles, there is a water vascular system controlling their turgescence. In acanthocephalans, the water-vascular system is reported to be related to the erection of the proboscis (Hamann, 1891; Rauther, 1930).

In the polychaete *Arenicola,* burrowing depends on hydraulic pressure in the body cavity (Chapman and Newell, 1947). Pentastomids seem to be able to set up relatively high body fluid pressures, used for the extension of their parapodia and other appendages (Heymons, 1926). In priapulids, the evagination of both the proboscis and the caudal appendage is due to body fluid sent under pressure into the parts to be evaginated (Fischer, 1925). Bryozoa possess a hydraulic pump mechanism which serves the evagination of their tentacles (Delage and Herouard, 1897). In nemerteans the protrusion and reversion of the proboscis is brought about by coelomic fluid pressure, set up by the contraction of the muscular proboscis, while retraction of the proboscis is largely due to contraction of the retractor muscles (Böhmig, 1929). A similar mechanism of proboscis eversion and retraction exists in sipunculids (Baltzer, 1934).

In the foregoing examples, body cavity fluid or water vascular fluid is responsible for hydraulic action. Examples of hydraulic action of vascular blood are also reported. Polychaete and oligochaete annelids which dig channels in earth or sand (*Arenicolida, Glassoscolecida, Megaloscolecida, Lumbricida*) possess a strongly developed vessel running laterally along the oesophagus (vas extraoesophagale)

(Fuchs, 1906). Brücke (1925, p. 852) believes that turgescence of this vessel might favor digging. The leech *Herpobdella* has a capillary net on both its anterior and posterior suckers. When suction occurs, blood from the neighboring vessels streams into the capillaries, thus taking part in the mechanical effect (Emden, 1929). Also in *Herpobdella* the spermatophores are squeezed out during the sexual act by an increase of blood pressure in a specialized organ. In sexual function the hydrostatic pressure of blood is often used mechanically: e.g. for penis erection in mammals. In pelecypod molluscs the swelling of the foot is due to hydraulic action of the blood (Buddenbrock, 1939). The stretching of the ocular tentacle of the common snail is based simply on relaxation of the retractor muscle. The existing blood pressure is sufficient to extend the tentacle (Buddenbrock, 1939).

These examples suffice to show the widespread use made of body fluids for hydraulic work.

Materials and Methods

Phascolosoma gouldi was furnished through the kindness of the supply department at the Marine Biological Laboratory in Woods Hole, Mass. *Sipunculus nudus,* on which most of the work was done, was collected on the beach of Morgat, Brittany.

The *Phascolosoma* used were 6 to 10 cm. long, the *Sipunculus* sometimes reached a length of 18 cm. in the expanded state.

Pressure was recorded by inserting straight capillary glass tubes into the coelom of the sipunculid as used by Inada (1947) in crayfish and mussels. Capillaries with a bore of 1 to 1.5 mm. were selected and their zero point determined. The pressures are expressed in cm. of coelomic fluid. Temporary withdrawal of fluid from the body cavity into the capillary did not affect the animals. To obtain a coordinated record of pressures and body movements a method of registration on a kymograph was used. The moving meniscus in the capillary tube was closely followed by means of a horizontal metal thread which was connected through a lever system with a writing point, applied against the drum of the kymograph. While one person recorded pressures, another person inscribed on the kymograph record, by a system of conventions, the movements observed on the animal.

Discussion

Types of movement. In *Sipunculus,* three types of movement may be distinguished: swimming, defense movements, and burrowing.

Swimming is observed frequently in freshly collected specimens kept in sea water without sand. In the regular, beat-like bendings of the body all circular muscles are contracted, while the longitudinal muscles of the dorsal and ventral sides contract and relax alternately (Uexküll, 1903). Swimming movements are accompanied by alternate rises and falls in pressure.

When strongly excited, *Sipunculus* and *Phascolosoma* execute defense movements. The two extremities of the body may be brought together and often cross, while the body of the animal turns around its longitudinal axis. The pressure, during this action, is considerably higher than during swimming movements. In

Phascolosoma it reaches commonly 50 cm., in *Sipunculus* commonly 70 to 80 cm. of body fluid.

Usually the defense reaction does not consist in active movements, but in a state of immobility accompanied by high turgor and the reduction of the body surface. The longitudinal muscles contract to their maximum. In both *Sipunculus* and *Phascolosoma* pressures reach top values around 100 cm. of body fluid. There seems to be no relation between maximum pressure and body size. The highest pressure observed—108 cm.—was in a small specimen of *Phascolosoma.*

Burrowing type movements and corresponding pressures. On animals put in a glass jar filled with sea-water and devoid of sand, five to six phases may be distinguished in the cycle of burrowing type movements (*Sipunculus*):

1) At the start there is formation of a "hump," i.e., a bending upwards of the central part of the body in a nearly vertical plane. At the same time the pressure begins to rise from initial values of 2–3 cm. of water.

2) While the hump is maintained, the eversion of the proboscis begins speedily. The pressure continues to rise.

3) The proboscis is partly protracted (usually about two-thirds) while the hump is being released. From that instant on the progression of the proboscis is slower. Simultaneously the pressure, reaching a maximum, mostly between 10 and 30 cm., stops rising, and starts falling immediately. The fall is usually completed before the next phase begins.

4) The tentacles appear. They are set free by a slight recession of the anterior extremity of the proboscis. Pressure remains constant, or, sometimes, drops very little.

5) Tentacles and proboscis are being retracted. During this action, a further, very slight fall of pressure (2–4 mm.) takes place. This fall may fail to occur or set in only after completion of proboscis retraction. Pressure attains exactly or nearly the level at which it started at the beginning of the burrowing cycle.

6) A period of immobility, of constancy of pressure. This phase is usually skipped when the animal is in a state of activity. In that case, phase 1 succeeds phase 5.

When *Sipunculus* remains in its feeding posture, the cycle is interrupted after phase 4.

During fast burrowing, which is accompanied by no tentacular eversion, the pressure, in preparation for the next protraction, rises while the proboscis is being retracted. In that case, high pressure is already present when the retractor muscles suddenly relax. The proboscis is, as it were, shooting out against the sand in full force. Such cases are illustrated by Figures 1 and 2, drawn after kymograph records.

The formation of the hump is synchronized with rise in pressure and the disappearance of the hump with fall in pressure. On the other hand, rise and fall in pressure are not essentially related to the protraction and retraction of the proboscis, as might have been expected *a priori.* Indeed, hump and definite pressure rise may fail to occur, while the proboscis is protracted normally, although more slowly than under marked pressure (Fig. 3). In several instances, the pressure was seen to remain constant at 1 cm. of body fluid during the whole protraction-retraction

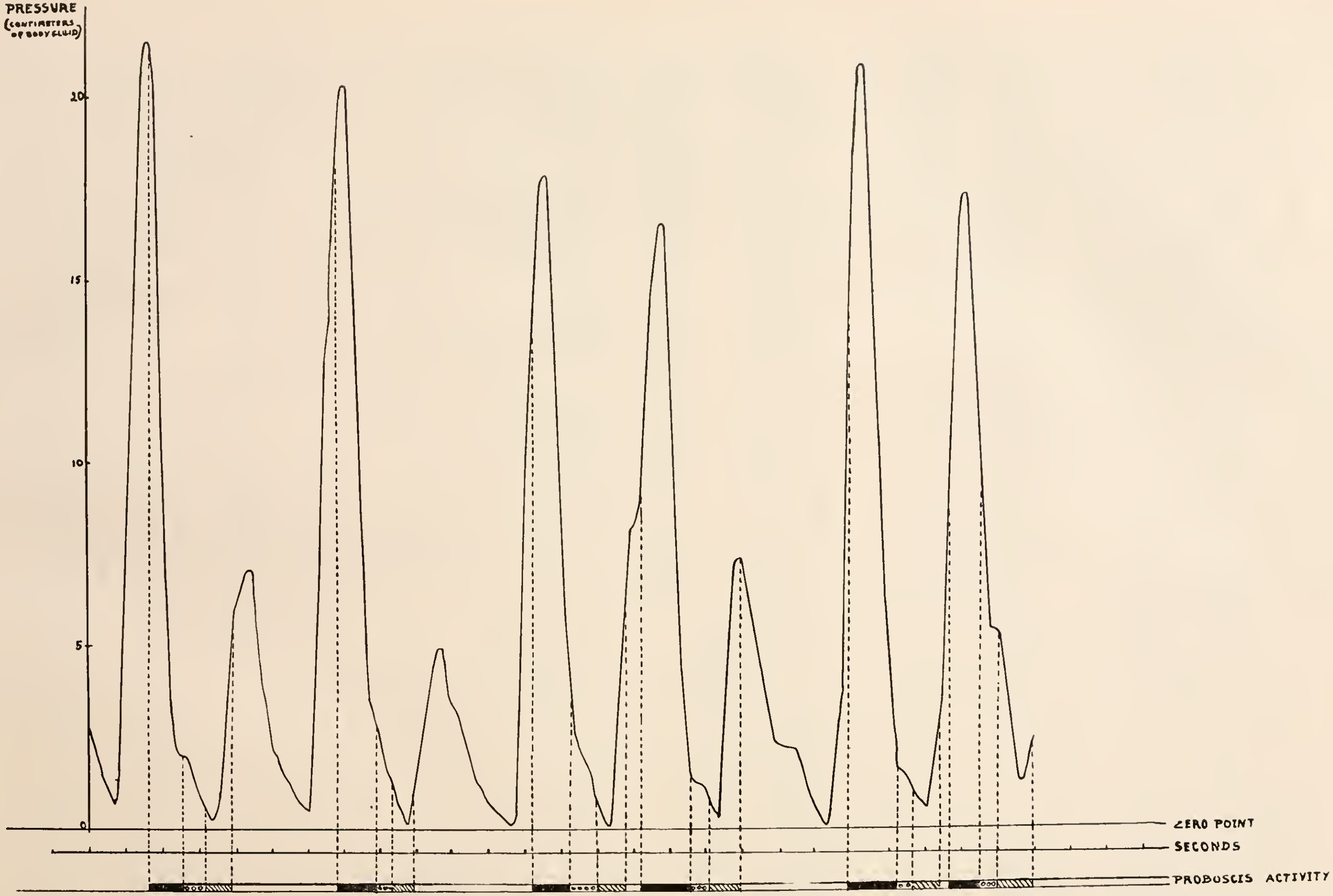

FIGURE 1. Data from kymographic record of coelomic fluid pressures in cm. coelomic fluid, and corresponding movements of proboscis of *Sipunculus nudus*. Time line: six second intervals. Proboscis activity indicated by following conventions: ■ proboscis being extended, ○○○○ proboscis extended, ▨ proboscis being retracted, □ proboscis retracted.

operation. However, protraction never occurred when the pressure was below 0.9 cm. of body fluid. The cessation of pressure rise and onset of fall may correspond to various states of protraction of the proboscis. Exceptionally the pressure rise may continue throughout the process of proboscis retraction. These irregularities illustrate the lack of strict coordination between pressure variations and proboscis protraction. Evidence of disjunction between the cycle of movements and the cycle of pressures is recorded by the kymograph record in Figure 1. There

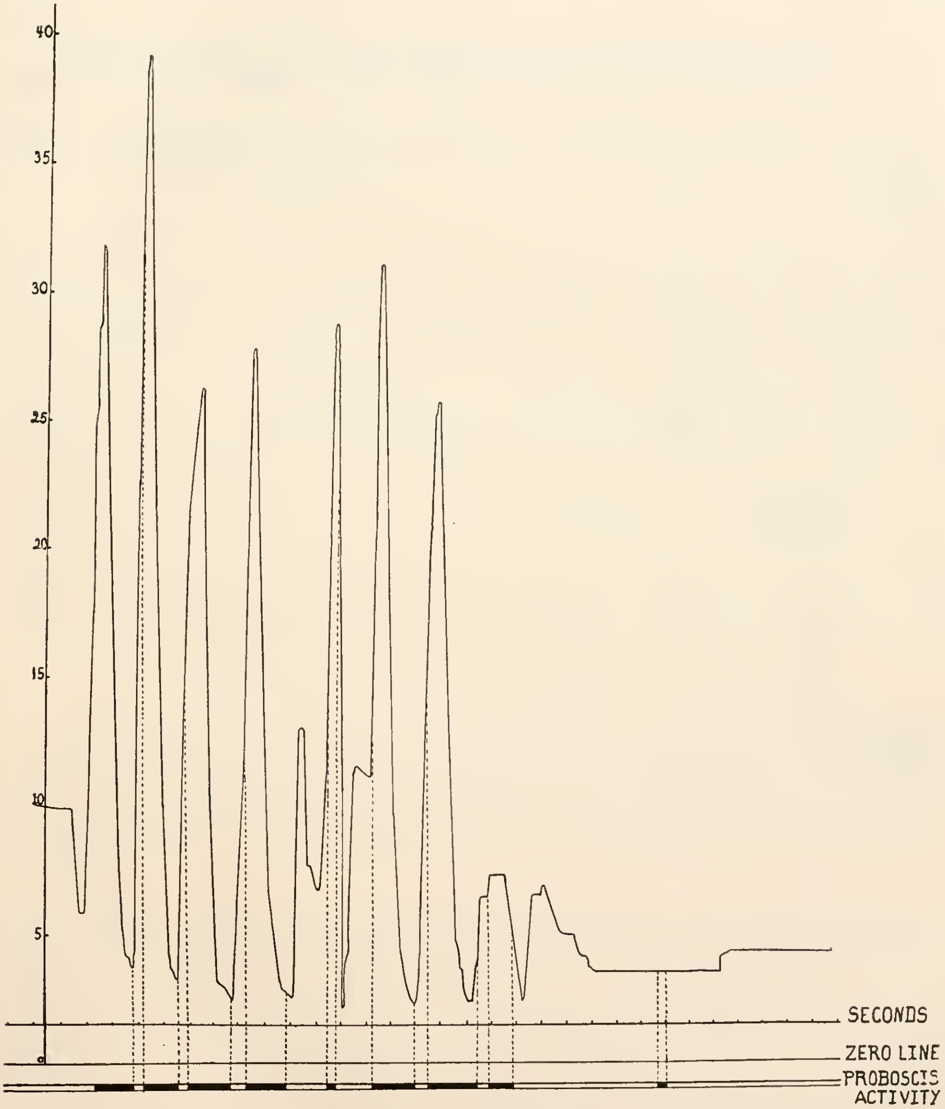

FIGURE 2. Pressure (ordinate) in mm. coelomic fluid in *Sipunculus nudus* recorded on kymograph. ▬ proboscis extending, ▭ all other phases of proboscis movements.

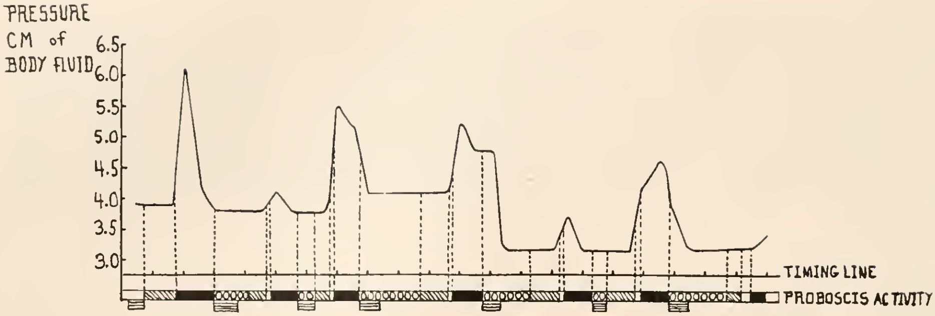

FIGURE 3. Pressure (ordinate) in coelomic fluid and activity in *Sipunculus nudus* recorded with aid of metronome beat every third second. Timing line gives 10 second intervals. Conventions for record of proboscis activity as in figure 1 with addition ▤ tentacles being extended.

we see, in several instances, one cycle of movements coinciding with two pressure cycles. The pressure cycle keeps firmly to its established timing, while the timing of the movement cycle is less constant; though keeping pace sometimes with the pressure cycle, at other times it is twice as slow. It should be noted that during what might be called an "extra-systole"—a pressure cycle not coordinated with proboscis protraction—the pressure never rises as high as it does otherwise. Even in this case a certain relation between movement and pressure is, then, maintained; very high pressures seem to be set up only when proboscis protraction really occurs.

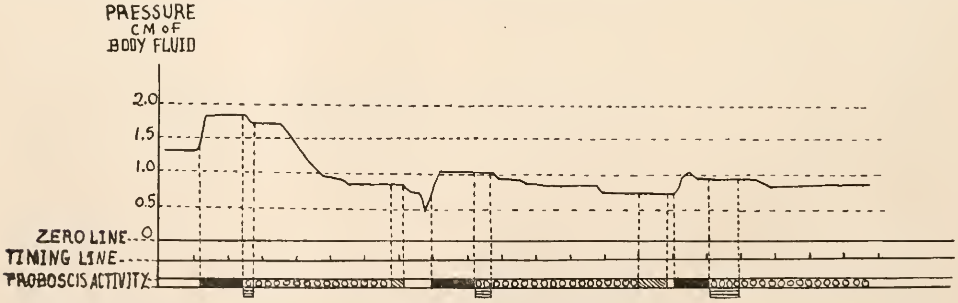

FIGURE 4. *Sipunculus nudus*, pressure and activity noted every fourth second with aid of metronome. Six second intervals on time line. Conventions for activity as in figure 3.

The 5th and 6th pressure rises in Figure 2 are illustrations of this. Here pressure has no time to drop to the usual minimum, before the rise accompanying protraction occurs and the movement cycle is not exactly half as slow as the pressure cycle. Thus the rhythmicity of the pressure cycle is upset to some extent. Depending on how soon after the "extra-systole" the large rise accompanied by proboscis protraction sets in, the "extra-systole" may be represented by a whole spike, as in Figure 1, or be reduced to a simple discontinuity of what appears to be one single pressure rise spike (6th spike on Figure 2).

The pressure rise may set in before the formation of the hump. In this case the appearance of the latter is characterized by a marked increase in speed of the rise.

The basic tonus, represented by the pressure minimum attained with phase 5, may remain constant during several consecutive burrowing cycles and then rise to a different level (Fig. 4).

The amplitude of pressure changes during burrowing movements in free water is very variable. In *Sipunculus* pressure during proboscis protraction was seen to attain maxima ranging from 1 cm. (Fig. 3) to about 40 cm. (e.g. animal I of table below). Maximum pressures registered during proboscis eversion in different specimens of *Phascolosoma* follow: 11.4; 12.4; 15.0; 16.0; 17.3; 21.3; 25.0; average: 16.9 cm.

The following table gives the relation between average pressure extremes and intervals between onsets of pressure rise in eight specimens of *Sipunculus*.

Animal	Average time elapsing between onset of two pressure rises (seconds)	Number of cycles averaged	Highest pressure reached (cm. of body fluid)	Average pressure minimum (cm. of body fluid)
I (Fig. 2)	12.7	8	39.6	2.6
II (Fig. 1)	15.6	11	21.2	0.4
III	17.3	6	20.1	2.5
IV (Fig. 4)	18	6	6.1	3.6
V	18	3	8.7	2.3
VI	18.4	6	25–30	—
VII	18.5	5	23.4	0.9
VIII	20.5	7	24.2	2.5

From these eight experiments, the average time elapsing between two pressure rises is about 17 seconds. The shortest time intervals are obtained in animal I which also showed the highest pressures, but in those with low maximum pressures the time intervals remain near the average (IV, V). The rhythmicity of pressures therefore seems largely independent of their magnitude. During most experiments the minimum pressures were nearly constant.

Proboscis eversion in *Sipunculus* occurs whatever the position of the animal, and not only, as Baltzer (1934) claims, when it lies on its ventral surface.

Pressures and movements during burrowing in sand. Specimens of *Sipunculus* were placed in a glass aquarium filled with sand, between a glass plate and the aquarium front. Since little space was available for lateral movements, the worm was often forced to slip along the aquarium front and its movements could thus be watched.

At the start of burrowing, pressures were similar to those given in the preceding section. As the animal gradually penetrated into the sand, the maximum pressures increased very considerably, the minimum pressures slightly. The increase in maximum pressures was sometimes strikingly gradual (Fig. 5). As long as only part of the animal's body was under sand, the maxima did not exceed those commonly observed during digging type movements in free water. Once the animal was about two inches below the surface, maximum pressures around 90 cm. were commonly reached (top value on record: 96 cm.). During these highest pressures greatest speed of progression was obtained. When the animal returned to low speed progression, the pressure maxima decreased markedly. Under comparatively low pressures the progression was never fast. High speed progression is not possible before the animal is wholly under sand. Indeed, the "hump" is effective under these conditions only as explained below. Since fast progression requires high pressure and is possible only when the animal is nearly completely under sand, pressure gradually increases as it becomes effective.

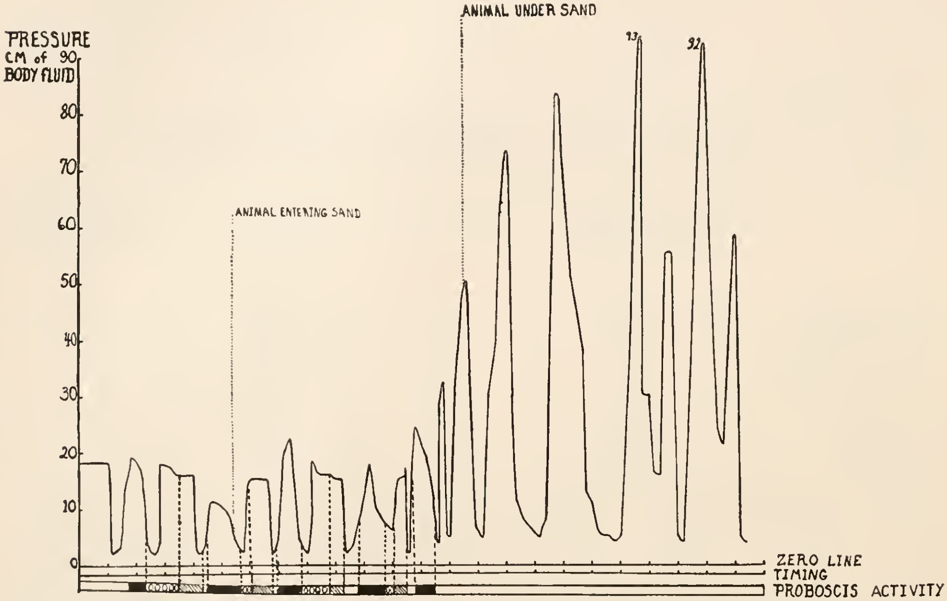

FIGURE 5. *Sipunculus nudus*, coelomic pressure and activity recorded every third second with aid of metronome. Conventions for proboscis activity as in figures 1 and 3. Time line, 10 second intervals.

Figure 6 shows how pressure variations and body movements are coordinated during burrowing under sand. The results are similar to those for burrowing movements in water except that the maxima often reach very much higher values. The period of low maxima corresponds to one of lower burrowing activity; the animal progresses less speedily and remains longer in the "feeding position" (proboscis and tentacles stretched out).

Under sand, proboscis protraction has been seen to continue under a pressure as low as 3.2 cm.

As in animals kept in free water, tentacle eversion was brought about by a retraction of the distal section of the proboscis. In this way the tentacles are set free within a cavity prepared beforehand by the burrowing proboscis and are protected from rough contacts with the sand.

Figure 5 affords evidence that the normal correspondence between burrowing cycles and pressure cycles may be systematically upset during burrowing in sand, just as it was occasionally during movements in free water. One burrowing cycle consistently corresponds to two pressure cycles. (The movements could not be followed during the later part of the record.) Proboscis retraction does not occur around the time of a pressure minimum, as in the case shown by Figure 1, but coincides with the top of a pressure spike. Curiously, retraction occurring at the moment of a pressure maximum seems to prevent the pressure from falling. A pressure plateau is maintained near the maximum as long as retraction lasts. The pressure spikes are always influenced by changes in the proboscis cycle, yet the rate at which the spikes follow each other may not be. Thus the two cycles (pressure and proboscis movements) may occupy various positions in time with respect to each other.

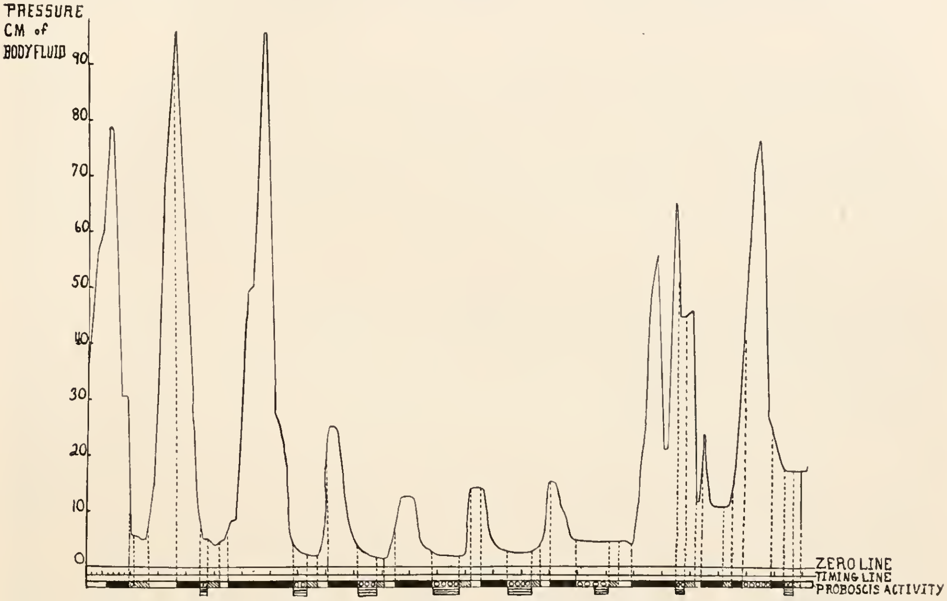

FIGURE 6. *Sipunculus nudus.* Coelomic pressures and proboscis movements recorded with aid of metronome every third second. Conventions as in figure 3. Time intervals, 10 seconds.

In conclusion, the motor nerve centers controlling contraction of the body wall appear to some degree independent of those for the movements of the proboscis, i.e., contraction and relaxation of the retractor muscles.[1] Moreover, pressure is likely to have a function partly independent of proboscis protraction.

The function of pressure during burrowing. With respect to its function, one may consider pressure from three points of view:

1) pressure necessary for proboscis eversion in absence of any resistance.
2) pressure necessary for the proboscis to overcome the resistance of the milieu.
3) pressure necessary to insure the progression of the animal in its milieu.

1. When the proboscis does not meet with any resistance, it can be protracted, as stated above, under exceedingly low pressure (minimum 0.9 cm.). Low pressure eversion differs from eversion under high pressure by being slow and very regular. A given low pressure is sometimes maintained although, with proboscis eversion, the

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[1] The muscles of the proboscis wall are not important in protraction. See below.

volume of the body cavity gradually increases. Thus is brought out the ability of the relaxed *Sipunculus* muscle to compensate exactly for any variation in pressure by corresponding variations of its length, so as to maintain a constant state of tension.

In the absence of coelomic pressure, proboscis protraction was never observed. An active animal was cut open along the dorsal midline, so as not to injure its nerve cord. Thus deprived of the possibility of setting up pressures, it was put back into sea-water. Although the muscles of the animal remained able to display activity, no eversion of the proboscis occurred. Therefore the presence of a head of pressure of at least 0.9 cm. of body fluid seems to be the necessary condition for proboscis eversion.

The question was raised whether this condition is also a sufficient one. In other words, do the muscles of the proboscis play any active part in eversion? The body of a specimen of *Sipunculus* was cut through transversely at the level of the anus and the posterior part of the worm discarded. The anterior part was kept immersed for several minutes in a solution of iodo-acetic acid (M/50), supposed to interfere with muscular contraction. Then the proboscis was drawn back by pulling on the retractor muscles. The fragment of the animal was slipped over the tip of a wooden rod and kept under sea-water. The system was made nearly water-tight by tying a thread over the cut end of the worm stretched over the stick. A capillary tube was introduced behind the base of the proboscis for measuring pressure, and sea-water was injected into the body cavity with a syringe. At 1.1 cm. of coelomic pressure (measured in cm. of sea-water), the proboscis was seen to unroll very slowly, part way. As the pressure rose to 1.7 cm., the proboscis extended quickly and thoroughly. The tentacles came out at the end of its eversion. Since the muscles of the proboscis poisoned with iodo-acetic acid did not display any activity, it is probable that active participation of the proboscis muscles is not needed for proboscis eversion and that very low pressure is indeed sufficient to bring about this eversion.

What, then, is the role of the proboscis muscle? It seems to be at least two-fold. One function is the retention of the proboscis inside the body-cavity. The muscles of the proboscis seem indeed able to help the retractor muscles, if not to replace them in that role. In animals whose retractor muscles had all been cut, the proboscis still resisted pressure and did not unroll. A second role of the proboscis muscles must be related to the "thixotropic" effect of digging, which will be discussed below.

2. We shall deal now with the pressure necessary to overcome the resistance of the sand.

The experiment reported above (setting up measured pressure in the body cavity of an anterior portion of a *Sipunculus,* the proboscis muscle being kept from contracting) was repeated on the same fragment of *Sipunculus* under 2 cm. of sand. Pressure was raised up to 9 cm. of sea-water, while the proboscis protruded only very slightly and was unable to continue its eversion.

On the other hand, in a normal animal burrowing in sand, proboscis eversion was observed, in one instance, as reported above, under a pressure of only 3.2 cm. of body fluid, and in a number of instances under slightly higher pressures.

These two results seemed inconsistent. And *a priori* it would not have seemed reasonable to assume that pressures as low as 3.2 cm. could overcome the resistance of the sand. It was impossible to drive out by pressure, under sand, the inverted finger of a rubber glove filled with water. The finger of a rubber glove surely meets a much higher resistance than the slender proboscis of *Sipunculus*. Nevertheless eversion of the proboscis in sand under a pressure of 3.2 cm. seemed hardly understandable.

The work of Chapman and Newell (1947) appears to offer the clue to this question. These authors, studying pressures in *Arenicola*, showed that very low pressures are sufficient for penetration in muddy sand. This kind of sand, which also is the medium of *Sipunculus*, behaves as a thixotropic colloidal system. "If the sand is prodded several times with a stick its resistance diminishes, and it remains in a semi-fluid state as long as the prodding continues." With an anesthetized proboscis or the water-filled finger of a rubber glove this thixotropic effect cannot be obtained, hence the failure, in these cases, to obtain protraction in sand under low pressure. However, the extending proboscis of *Sipunculus* probably has an effect similar to the prodding of a stick, and in this respect the muscles of the proboscis must play an outstanding rôle.

3. The function of high pressure, unnecessary to proboscis eversion even under sand, seems to be to insure efficient progression of the worm in the sand. Large pressure rises have been reported always to be accompanied by a bending of the central part of the body. This bending also occurs under sand, as far as compatible with the resistance of the walls of the burrow. The central part of the worm is thus immobilized on a given spot by the pressure exerted against these walls. The body is therefore prevented from being pushed back during the action of proboscis protraction. At the same time, the high pressure insures a speedy progression of the proboscis, thus increasing the "prodding" effect. High pressure seems therefore to have a triple role: to maintain the hump, which serves as anchorage in the burrow; to favor the thixotropic action of the proboscis; and to insure speedy progression.

One may wonder how the animal can do without the hump during the last slow phase of proboscis eversion. It should be remembered that the hump is not in all circumstances a necessary condition for proboscis progression. Indeed, when the proboscis first penetrates into the sand, the rest of the body, including the hump, is still outside it. Nevertheless the animal is able to progress slowly.

Yet during the more energetic phase of burrowing, the hump, no doubt, affords a necessary fulcrum. It corresponds to the "collar," a swelling appearing on the distal extremity of the proboscis. It is assumed that, due to a grip on the sand at this swelling, the body can be drawn forward.

The hump has another important function. It creates on one side a hollow space between the wall of the burrow and the animal's body. In this space a backward flow of sand particles was consistently observed. Apparently the sand stirred up by the proboscis during the first phase of its protraction is thus flowing back, leaving ahead the space necessary for the progression of the body. The body has indeed a much larger diameter than the proboscis and could not follow the proboscis, if the latter burrowed a channel of just its size. The effects on the sand of the thixotropic stirring action of the proboscis were thus directly observed, and in relation to them the hump appears to be indispensable. Considering the resistance of the walls of the burrow, this hump can develop in sand under high pressure only.

The necessity of high pressure for progression in sand was shown by Uexküll (1903). He destroyed in a specimen of *Sipunculus* the posterior part of the nerve cord. The posterior part of the body was distended, high pressure could not be developed and the animal was not able to progress in the sand. If Uexküll then cut off the paralyzed posterior section and closed the opening with a rubber stopper, the anterior half of the worm was seen to disappear in the sand within a few minutes.

The role of pressure in circulation of coelomic fluid. Circulation of coelomic fluid in *Sipunculus* is brought about by several factors: the ciliation of the coelomic epithelium (Mayer, 1929), the stirring action of the urns—little ciliated organelles swimming around freely in the body cavity—and the movements of the body wall. The effect of cilia is probably the only one acting consistently in the sense of fluid movement in a closed circle throughout the body.

It is clear that a mechanical action on body fluids cannot be qualified as being "circulatory" in nature, unless it shows some rhythmicity and constancy during at least a certain length of time. The question was raised whether digging, constituting one of the few rhythmical activities of the sipunculid, may have a sufficiently regular influence to cause circulation of coelomic fluid.

In order to study the effects of pressure variation during burrowing movements from this point of view, a few small air-bubbles were injected into three specimens of *Sipunculus.* The animals were placed on a glass plate over a light so that the bubbles were visible. Care was taken to keep the animals in horizontal position. A regular movement of air bubbles during proboscis movements was observed in the anterior region of the body only. At the start of proboscis eversion the bubbles were shifting anteriorly, at the start of retraction caudally. Few shifts were observed in the middle and posterior regions. It is concluded that during burrowing movements, significant pressure gradients are rhythmically established in the front region only. They have an effect on the movements of coelomic fluid.

Some simultaneous measurements of pressure in the anterior and posterior part of the body were compatible with the air bubble experiment, the pressures in the posterior capillary rising and falling faster than those recorded by the anterior capillary.

SUMMARY

The relationship between coelomic pressures and body movements has been investigated in *Phascolosoma gouldi* and, mainly, *Sipunculus nudus.*

During "defense movements," pressure in *Phascolosoma* is commonly 50 cm. of water, in *Sipunculus* 70 to 80 cm. During "defense immobility," the highest pressure recorded, in *Phascolosoma,* was 108 cm. There does not seem to be a relation between body size and maximum pressure.

Six phases of burrowing movements have been distinguished in *Sipunculus* and related to coelomic pressure. An important pressure rise coincides with a bending movement of the central part of the body. This "hump" and the high pressures associated with it have functions which are described. Very low coelomic fluid pressures suffice to cause eversion of the proboscis. Pressure is a necessary and sufficient condition for proboscis eversion in free water, while in sand the proboscis muscles seem to play an essential role during proboscis eversion in relation to the thixotropic property of stirred sand.

During most experiments, minimum pressures were very constant, while maximum pressures varied a great deal. During burrowing movements in free water, the highest pressure recorded in *Phascolosoma* was 25.0 cm., in *Sipunculus* 39.6 cm. of body fluid. During burrowing in sand, pressure of 96 cm. water was reached by *Sipunculus*.

The frequency of pressure cycles seems to be independent of the absolute pressure.

The coordination between the cycles of pressure and proboscis movements is variable. Therefore the retractor muscles and the muscles of the body wall are probably dependent on nervous centers which are not closely associated.

Pressure variations during burrowing have an influence on circulation of coelomic fluid.

Acknowledgment

I wish to express my gratitude to Professor C. Ladd Prosser for directing my interests to pressure relations among invertebrates and for editing the manuscript.

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METABOLIC ACTIVITIES AND CLEAVAGE OF EGGS OF THE SEA URCHIN, ARBACIA PUNCTULATA

A REVIEW, 1932–1949[1]

M. E. KRAHL

Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri, and the Marine Biological Laboratory, Woods Hole, Massachusetts

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[1] This review was prepared at the invitation of the Editorial Board.

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A. INTRODUCTION

The literature on the chemical and physical properties of eggs of *Arbacia punctulata* was summarized by Harvey (1932). During the ensuing period, the use of eggs of Arbacia and other sea urchins as representative cells for the study of the fundamental properties of animal tissue has greatly increased.

Two problems, closely related to each other, have been extensively studied: the analysis of the chemical events associated with fertilization; and the elucidation of the chemical processes by which energy liberated by oxidation of foodstuffs is made available for mitotic processes and cleavage.

The first of these two problems is the subject of excellent reviews by Tyler (1948) and Runnström (1949) and will therefore be dealt with here only incidentally.

The second problem is constantly gaining in interest because of its fundamental relationship to normal and pathological growth processes. As the result of investigations by both American and European workers there is probably no cell or tissue, with the possible exception of skeletal muscle, for which information relating enzymatic processes to function is as complete as it is for the sea urchin egg.

The principal objective of this paper is to bring together what is known about the relation of metabolic enzymes and metabolic processes to cleavage in the Arbacia egg. In addition, because of their potential bearing on this objective, the effects of chemical agents upon metabolic activity and cleavage are recorded; those agents which have known effects upon enzymes are stressed, but other agents are included. Chief attention will be given to the years 1932–1949 inclusive, publications prior to 1932 having been covered in the aforementioned review by Harvey (1932). Observations upon other marine eggs will be introduced where a comparison with the results on Arbacia is desirable.

Every effort has been made to leave no significant aspect of the relation of metabolic processes to cleavage unmentioned, but the bibliography does not include every publication in this field, as a given set of observations has in many instances been published in more than one place.

B. OXYGEN CONSUMPTION

1. UNITS. Oxygen uptake of marine eggs has usually been expressed as c. mm. per 10^6 eggs or as c. mm. per hour per 10 c. mm. eggs. To permit expression of results in various units, the following data are useful.

Egg dimensions, average diameter, 74 μ; surface, 17,200 μ^2; volume, 212,000 μ^3 (Harvey, 1932). By diffractometer method, the mean volume is 228,000 μ^3 (Korr, 1937).

Density, 1.09 (Harvey, 1932).

Dry weight, 24.2 per cent of wet weight (Ballentine, 1940a); 23.9 per cent (Hutchens et al., 1942).

Nitrogen content, 0.107 mg. nitrogen per mg. dry weight, 5.86 mg. nitrogen per 10^6 cells (Ballentine, 1940a); 0.101 mg. nitrogen per mg. dry weight, 5.93 mg. nitrogen per 10^6 cells (Hutchens et al., 1942). Egg protein is assumed to be 6.25 times the egg nitrogen content.

Cells per mg. dry weight, 17,600.

Cells per 10 c. mm., 46,500.

Cells per wet gram, 4,300,000.

Weight of 10 c. mm. eggs, 10.9 mg. wet, 2.63 mg. dry.

2. DETERMINATION OF EGG VOLUME. Measurements of egg volume have been commonly made by the hematocrit method. The hematocrit values obtained depend upon the period and intensity of centrifugation, and upon whether the volume which is measured is the total volume (eggs plus sedimentable jelly) or egg volume only. The most frequently used hematocrit value is that for the eggs only, as determined by centrifuging 5 minutes or more at 2000 × gravity or above, i.e. to an essentially constant volume. The egg volume as thus determined by hematocrit is about 8 per cent larger than calculated from hemocytometer measurements (Shapiro, 1935; Clowes and Krahl, 1936). The volume of a given number of eggs is 8 per cent smaller after fertilization than before (Glaser, 1924).

When results are expressed in terms of egg volume, it is helpful to state whether volume is determined on fertilized or unfertilized eggs.

3. OXYGEN CONSUMPTION OF WHOLE EGGS. Measurements on oxygen consumption of whole eggs are recorded in Table I.

The rate of oxygen consumption is strongly dependent upon the degree of trauma to which the Arbacia eggs are subjected prior to or during the measurement of oxygen consumption. Shaking rates above 48 per minute at an amplitude of 7.5 cm. (Whitaker, 1933a), light centrifuging during washing of eggs (Velick, 1941) or any other treatment which tends to break down the egg surface layers produces an increase in rate of oxygen consumption. Tyler, Ricci, and Horowitz (1938) found that the rise in oxygen consumption of unfertilized eggs, which begins some five hours after the eggs are shed, does not occur when the bacterial population in the sea water around the eggs is not allowed to increase.

For these reasons the absolute value of the oxygen consumption of *Arbacia punctulata* eggs cannot be specified except in relation to the conditions used for measurement. It follows that measurements designed to show effects of chemical agents and other treatments should be made under very carefully controlled and precisely described basal conditions. When the experimental conditions are kept constant, the values are reproducible from season to season.

Increase in oxygen consumption upon fertilization. The change in oxygen consumption of *Arbacia punctulata* eggs upon fertilization has been repeatedly studied since Warburg (1908) first showed oxygen consumption of the *Arbacia pustulosa* egg to be increased upon fertilization (Loeb and Wasteneys, 1911; Tang and Gerard, 1932; Whitaker, 1933a; Rubenstein and Gerard, 1934). The last named authors found that the factor by which oxygen consumption increases upon fertilization is a variable, its magnitude depending upon temperature. At 11° C., the fer-

TABLE I

Oxygen consumption of unfertilized and of fertilized eggs of Arbacia punctulata in natural sea water. The measurements are tabulated in chronological order. Where conversion factors were necessary, those given earlier in this paper were applied to the original measurements. A discussion of the validity of the numerical values is given in the text.

Observer	T° C.	Fert. (F) or unfert. (U)	Period of measurement, time after fertilization	Oxygen consumption
			hours	c. mm. per hr. per 10 c. mm. eggs
Tang (1931)	24.7	U	—	1.6 [1,2]
		F	0.25–1	7.9
Tang and Gerard (1932)	25	F	0.25–1	5.5 [1]
Whitaker (1933a, 1935)	21	U	—	0.4–0.5
		F	0.38–0.63	2.0
Gerard and Rubenstein (1934)	21	U [3]	—	0.9
Shapiro (1935a)	25.9	U	—	1.5
		F	—	3.9
Clowes and Krahl (1936)	20	U	—	0.6–0.7
	12	F	0.42–3	1.7
	20	F	0.42–3	3.1
	27	F	0.42–3	5.5
Korr (1937) [4]	15	U	—	0.2
	20		—	0.5
	25		—	1.0
	12	F	—	1.7
	15		—	2.1
	20		—	3.0
	25		—	4.7
Tyler, Ricci, and Horowitz (1938)	20	U	—	0.7
Krahl and Clowes (1938a)	20	F	0.42–3	3.1
Hutchens, Keltch, Krahl, and Clowes (1942)	20	F	1–4	2.4
			4–7	4.4
			7–11	4.9
			11–16	5.9
			16–26	6.8
Keltch and Clowes (1947)	20	F [5]	—	4.8

[1] The eggs were centrifuged lightly during washing, which tends to raise the oxygen consumption.

[2] Values are also given for various partial pressures of oxygen.

[3] Data are also given for change in oxygen consumption upon fertilization at temperatures from 11–29.9° C.

[4] The effects of pyocyanine, cyanide, and combinations of the two were also measured over the temperature range 11–26° C.

[5] Artificial parthenogenesis by hypertonic NaCl; 98 per cent membrane formation, 18–45 per cent cleavage.

tilized rate is about ten times the unfertilized; at 29.9° C., it is only twice that of the unfertilized.

At 20° C., the increase in oxygen consumption of Arbacia eggs is as large after artificial parthenogenesis (hypertonic NaCl) as after sperm fertilization (Keltch and Clowes, 1947). Mechanisms by which the increase in oxygen consumption upon fertilization may be brought about have been discussed by Whitaker (1933b), Krahl, Keltch, Neubeck and Clowes (1941), Goldinger and Barron (1946), Runnström (1949), Korr (1937), Ballentine (1940), and others. Whitaker (1933b) has summarized the measurements upon oxygen consumption of eggs of other marine forms: *Asterias forbesii, Cumingia tellinoides, Chaetopterus, Fucus vesiculosus, Nereis limbata.*

Oxygen consumption of the Arbacia egg increases gradually after fertilization until the egg is hatched, and then more markedly (Hutchens *et al.*, 1942). Similar studies on fertilized eggs of European sea urchins have been made by Gray (1927), Lindahl and Holter (1941), and Borei (1948). Zeuthen (1947) reported that, during the 5th to 8th cleavages of Psammechinus, the oxygen consumption is higher in the prophase, and lower in the later phases of a given mitotic cycle.

4. Oxygen Consumption of Egg Fragments. By high speed centrifuging in solutions of suitable specific gravity, unfertilized Arbacia eggs may be broken into a light half, containing the nucleus, the mitochondria, and some yolk; and a heavy half, containing the pigment granules and the major portion of the yolk. It has been found that the oxidative activity of the heavy half is somewhat greater than that of the light half, whether measured by oxygen consumption (Shapiro, 1935) or as reducing activity toward ferricyanide (Ballentine, 1940c). The combined oxygen uptake of the two halves of the unfertilized egg is 29 per cent greater than that of the whole egg, when proper allowance was made for volumes of the fragments; the sum of the respiratory rates of the two kinds of fertilized halves is 17 per cent less than that of the whole fertilized egg. Addition of p-phenylenediamine to the light halves produces a relatively smaller increase in oxygen consumption than addition of the same agent to the heavy halves (Boell *et al.*, 1940).

5. Respiratory Quotient. The respiratory quotient of unfertilized Arbacia eggs has apparently not been measured. That of the fertilized eggs appears to vary with the batch of eggs used; the rate of glycogen disappearance is low when the respiratory quotient is low. Observed R.Q. values are, for various periods after fertilization (Hutchens, Keltch, Krahl, Clowes, 1942): 1–4 hours, 0.85 (0.76–0.94); 1–7 hours, 0.88 (0.75–1.00); 1–11 hours, 0.90 (0.75–1.04); 1–16 hours, 0.88 (0.79–0.97); 1–25 hours, 0.86 (0.84–0.98). The respiratory quotient of the *Arbacia pustulosa* egg was found to be: unfertilized, 1.06; fertilized, 0.95 (Ashbel, 1930).

6. Oxygen Consumption of Sperm. See Barron, Seegmiller, Mendes, and Narahara (1948); Benedict and Barron (1946).

C. Rate of Cleavage

The times from fertilization to 50 per cent cleavage at 20° C. were observed by Fry (1936) to be: first cleavage, 69 minutes; second cleavage, 107; third cleavage, 145. For times to first cleavage at other temperatures, and times to other stages of development, see Harvey (1932).

Effects of various physical or chemical agents upon cleavage rate have been measured by two methods. In one, employed first by Smith and Clowes (1924), and used by Clowes, Krahl, and coworkers for their measurements, the control and experimental aliquots of eggs are allowed to develop until the control eggs have undergone several divisions; the eggs are then fixed in 0.1 per cent formaldehyde, and the percentage of the total in each state of division is determined by counting. The extent of division is expressed numerically according to the number of divisions undergone: no division, 0; 2 cell, 1; 4 cell, 2; 8 cell, 3; 16 cell, 4. For example, a population 25 per cent 4 cells, 70 per cent 8 cells, and 5 per cent undivided would have undergone, on the average, $0.25 \times 2 + 0.70 \times 3 + 0.05 \times 0 = 2.6$ divisions per egg. Cleavage in presence of various agents can be expressed as a fraction of control rate by dividing the divisions per egg in the experimental sample by the divisions per egg in the control.

In the second method of determining cleavage rate, the time to reach 50 per cent first cleavage is determined by serial fixation and counting of samples. The effect of experimental conditions can then be expressed as minutes of cleavage delay (or acceleration), as ratio of time required for the experimental sample to that required for control, or as per cent delay or acceleration relative to the control rate.

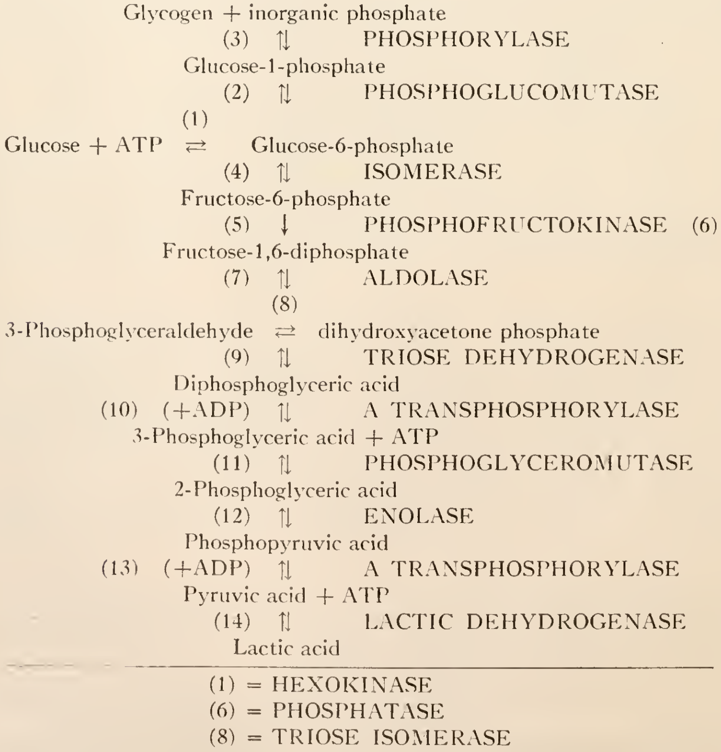
Glycogen + inorganic phosphate
(3) PHOSPHORYLASE
Glucose-1-phosphate
(2) PHOSPHOGLUCOMUTASE
(1)
Glucose + ATP
Glucose-6-phosphate
(4) ISOMERASE
Fructose-6-phosphate
(5) PHOSPHOFRUCTOKINASE (6)
Fructose-1,6-diphosphate
(7) ALDOLASE
(8)
3-Phosphoglyceraldehyde
dihydroxyacetone phosphate
(9) TRIOSE DEHYDROGENASE
Diphosphoglyceric acid
(10) (+ADP) A TRANSPHOSPHORYLASE
3-Phosphoglyceric acid + ATP
(11) PHOSPHOGLYCEROMUTASE
2-Phosphoglyceric acid
(12) ENOLASE
Phosphopyruvic acid
(13) (+ADP) A TRANSPHOSPHORYLASE
Pyruvic acid + ATP
(14) LACTIC DEHYDROGENASE
Lactic acid
(1) = HEXOKINASE
(6) = PHOSPHATASE
(8) = TRIOSE ISOMERASE

FIGURE 1. Steps in anaerobic breakdown of glucose or glycogen in animal tissues.

D. METABOLIC CATALYSTS, METABOLIC INTERMEDIATES; PHOSPHORUS, NUCLEIC ACID AND CALCIUM METABOLISM; ECHINOCHROME

1. METABOLIC CATALYSTS. The search for metabolic catalysts in the sea urchin egg has been guided by previous observations on yeast and skeletal muscle. According to current concepts, carbohydrate is apparently broken down by muscle in two phases; in the first (anaerobic) phase, glucose or glycogen is broken down to pyruvate or to a 2 carbon fragment (Fig. 1). In the second (aerobic) phase, the carbons of the pyruvate or 2 carbon fragment are converted to carbon dioxide via the reactions of the tricarboxylic acid cycle (Fig. 2). The hydrogens removed during the aerobic phase are transferred, over diphosphopyridine nucleotide or triphosphopyridine nucleotide, flavin-adenine dinucleotide, and the cytochromes to react finally with oxygen and form water (Fig. 3); the hydrogens removed during the anaerobic cycle are used to reduce pyruvate to lactate. Amino acids employed as foodstuffs are first deaminated and then oxidized over the tricarboxylic acid cycle. Fatty acids to be oxidized are broken down to 2 carbon fragments which then also enter the tricarboxylic acid cycle (see Sumner and Somers, 1947; Krebs, 1943; Ball, 1944; and Lardy, 1949, for further discussion of biological oxidations).

During the sequence of events summarized in Figs. 1–3 energy is liberated by oxidation of the foodstuff. It has been postulated with the enzymes of kidney or liver that some 60 per cent of the energy derivable by complete oxidation of carbohydrate can apparently be stored up in the form of so-called "high energy" phosphate bonds (Lipmann, 1941; Hunter and Hixon, 1949). The terminal group of adenosine triphosphate is such a bond and this group can be transferred to synthesize other more stable phosphate compounds or to create active intermediates in the synthesis

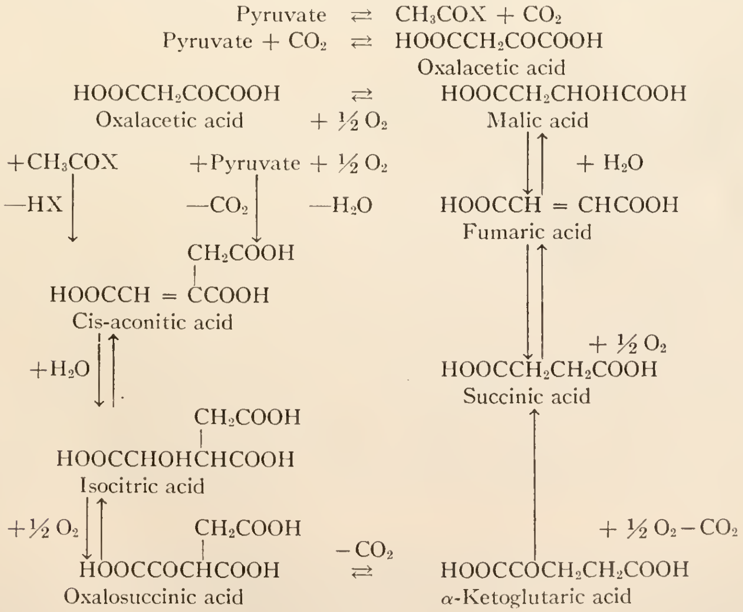
Pyruvate ⇄ CH3COX + CO2
Pyruvate + CO2 ⇄ HOOCCH2COCOOH
Oxalacetic acid
HOOCCH2COCOOH
Oxalacetic acid
+ ½ O2
HOOCCH2CHOHCOOH
Malic acid
+CH3COX
—HX
+Pyruvate + ½ O2
—CO2
—H2O
+ H2O
HOOCCH = CHCOOH
Fumaric acid
CH2COOH
HOOCCH = CCOOH
Cis-aconitic acid
+H2O
+ ½ O2
HOOCCH2CH2COOH
Succinic acid
CH2COOH
HOOCCHOHCHCOOH
Isocitric acid
+½ O2
CH2COOH
HOOCCOCHCOOH
Oxalosuccinic acid
– CO2
+ ½ O2 – CO2
HOOCCOCH2CH2COOH
α-Ketoglutaric acid

FIGURE 2. Some possible steps for pyruvate utilization via the tricarboxylic acid cycle.

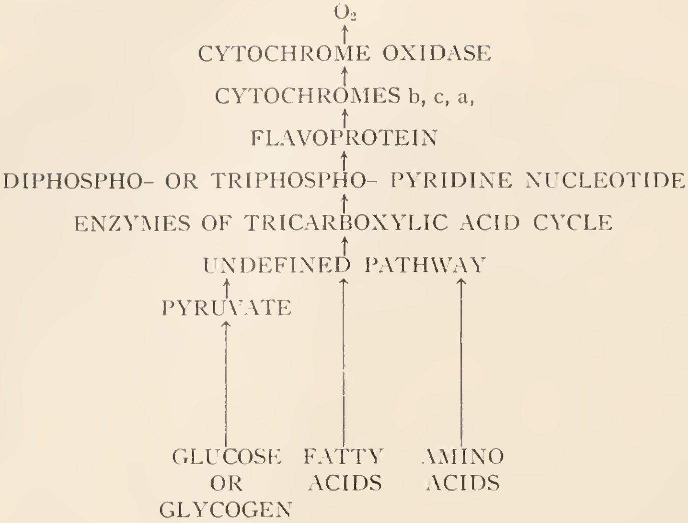

FIGURE 3. Schematic outline of steps by which hydrogens (or electrons) of foodstuffs react with oxygen in animal tissues to form water.

of many cellular constituents. From these facts it is postulated that the major portion of the energy used for synthesis and function must pass through such a mechanism. Most of these "high energy" phosphate bonds are created during the aerobic phase of substrate disposal mentioned above.

None of the enzymes for the anaerobic steps shown in Figure 1 has been studied in Arbacia eggs, nor have specific enzymes for deamination of amino acids or breakdown of fatty acids been looked for.

a. Cytochrome oxidase. Arbacia eggs contain an enzyme which is similar to the cytochrome oxidase of other animal tissues, in that it oxidizes reduced cytochrome c, the cytochrome c concentration for half maximal activity being 4×10^{-6} *M*. The enzyme is reversibly inhibited by carbon monoxide; it is also inhibited by low concentrations of other iron-binding reagents such as cyanide, azide, and sulfide; it is not inhibited by copper-binding reagents such as 8-hydroxyquinoline or diethyldithiocarbamate. This enzyme occurs in nearly equal amounts in unfertilized and fertilized Arbacia eggs, being present in both in a concentration sufficient to account for the respiration of the fertilized eggs even under the maximum respiratory stimulation yet observed (Krahl, Keltch, Neubeck, Clowes, 1941).

Measurements with the Cartesian diver upon centrifugally isolated fractions show the cytochrome oxidase of Arbacia eggs to be associated with the small particulate elements of the cytoplasmic matrix (Hutchens, Kopac, Krahl, 1942) rather than with the particles stainable with Janus green which are customarily referred to as mitochondria (Harvey, 1941).

b. Cytochromes and related compounds. No cytochrome c has been found in Arbacia eggs (Ball and Meyerhof, 1940; Krahl, Keltch, Neubeck, Clowes, 1941). The latter workers used a concentrating procedure which was shown to detect 2 micrograms of added cytochrome c per gram of eggs. This quantity would be equivalent to about $2 \times 10^{-7} M$ in the egg cytoplasm; since the measurements referred to above indicate that the cytochrome oxidase of the egg is fully active only at concentrations above $10^{-5} M$, and only 10 per cent active at $10^{-6} M$, considerable doubt arises as to a possible role for cytochrome c in the Arbacia egg.

Ball and Meyerhof found, in the Arbacia egg, iron porphyrin compounds related to the active group of cytochrome c. Cytochromes a and b have not been demonstrable on the basis of spectroscopic observations.

Cytochrome c is plentiful in Arbacia sperm (Ball and Meyerhof, 1940).

c. Flavin-adenine dinucleotide. Flavin-adenine dinucleotide has been extracted from Arbacia eggs and identified by its ability to serve as the prosthetic group of d-amino acid oxidase and by the quantitative absorption spectrum of the lumiflavin obtained from it (Krahl, Keltch, Clowes, 1940). The average content is about 10 micrograms per gram wet eggs, no consistent change with fertilization and development being noted.

d. Diphosphopyridine nucleotide (DPN). DPN has been extracted from the eggs with warm dilute acid, and assayed in terms of its ability to serve as the active group of phosphoglyceraldehyde and glycerophosphate dehydrogenases (Jandorf and Krahl, 1942). The apparent content of DPN in Arbacia eggs at various times after fertilization is, as micrograms per gram wet eggs: unfertilized, 385; fertilized 30 minutes, 345; fertilized 10 hours, 242. With modified extraction procedures, to preserve the acid labile (presumably dihydro-) form of DPN, the total DPN remains constant during the first 10 hours of development at about 300–400 micrograms per gram of eggs.

e. Enzymes of the tricarboxylic acid cycle. The experimental evidence currently available for mammalian tissues indicates that a cycle of reactions (similar to that illustrated in Fig. 2) may provide the mechanism by which the two carbon or three carbon fragments arising from partial breakdown of carbohydrate, amino acids, or fatty acids are converted to carbon dioxide with liberation of energy in a form which cells can use for synthetic reactions or for function. The structure of the initial condensation product arising from pyruvate and oxalacetate is not settled; it may be cis-aconitate, citrate, or possibly some other substance. Pyruvate is oxidized during a sequence of reactions to give three CO_2 molecules per pyruvate. One CO_2 is liberated during formation of the initial six carbon product of condensation, one during the oxidation of oxalosuccinic acid, and one during oxidation of α-ketoglutaric acid to succinic acid. By use of the enzymes from kidney, it has been demonstrated that about 15–20 atoms of inorganic phosphorus are converted into a "high energy" form during oxidation of each pyruvate, most, if not all, during and subsequent to the oxidation of α-ketoglutarate (Ochoa, 1943; Lehninger and Smith, 1949; Hunter and Hixon, 1949).

Efforts to demonstrate the role of the tricarboxylic acid system in Arbacia eggs have so far been confined to determining, first, whether pyruvate can be oxidized by the eggs and, second, whether oxalacetate, α-ketoglutarate, or succinate can be oxidized and, if so, whether "high energy" phosphate is generated thereby.

Oxidation of pyruvate. In most cells both the condensation and the decarboxylation reactions of pyruvate apparently require diphosphothiamine, the biologically active form of vitamin B_1 (Barron and Lyman, 1940).

The diphosphothiamine content of the Arbacia egg was assayed on the basis of the ability of this substance to serve as the active group of yeast decarboxylase. Both Krahl, Jandorf, and Clowes (1942), and Goldinger and Barron (1946) found Arbacia eggs to contain 2–3 micrograms diphosphothiamine per gram wet eggs; this value was not significantly altered by fertilization.

Goldinger and Barron (1946), using a chemical method for pyruvate estimation, found about 70 micrograms of pyruvate per gram dry weight of unfertilized or fertilized Arbacia eggs. They determined the rate of disappearance of pre-formed pyruvate in intact unfertilized and fertilized Arbacia eggs; the rates of pyruvate disappearance at 20° C. were found to be: unfertilized, 66 γ, fertilized, 456 γ, per hour per gram dry weight; these values correspond to 0.2 and 1.3 μM per hour per gram wet weight. Krahl, Jandorf, and Clowes (1942), using a specific but relatively less accurate enzymatic method for pyruvate estimation, determined the rate of disappearance of pyruvate added to cytolysates of unfertilized and fertilized eggs; their values, in μM per gram wet eggs per hour at 20° C., were: unfertilized, 1.1; fertilized, 1.5. It will be noted that the rates for the eggs and for cytolysates are nearly the same after fertilization, but that breakdown of egg structure has raised the rate for cytolysates of unfertilized eggs closer to the fertilized level.

Neither Goldinger and Barron nor Krahl, Jandorf, and Clowes determined the fate of the pyruvate or whether its oxidative removal was coupled with generation of "high energy" phosphate bonds.

Relation of oxidation of oxalacetate, α-ketoglutarate, and succinate to phosphorylation. A cell-free particulate system has been prepared from unfertilized Arbacia eggs which can use substrates of the tricarboxylic acid cycle to effect phosphorylation (Keltch, Strittmatter, Walters, and Clowes, 1949, 1950). Although the effect of these substrates on oxygen consumption is small, owing to a large baseline respiration, phosphorylation is specifically made possible by their oxidation. The phosphorylation is increased from 130 γ per hour in absence of added substrate to 1010 γ with α-ketoglutarate, 910 γ with oxalacetate, and 530 γ with succinate, all values being for a quantity of homogenate obtained from 1 gram wet unfertilized eggs. The ratio: atoms P esterified/atoms oxygen consumed was found to be 1.1 with α-ketoglutarate as substrate.

To prepare a cell-free particulate system capable of oxidizing these substrates, it is necessary to minimize breakdown of the pigment granules during homogenization, as echinochrome apparently inactivates one or more of the catalysts required (Crane and Keltch, 1949). Echinochrome is a substituted naphthoquinone (see below) capable of oxidizing reduced sulfhydryl groups which are known to be essential for oxidation of succinate and other substrates (Hopkins and Morgan, 1938; Barron and Singer, 1943). Earlier unsuccessful attempts to demonstrate oxidation of α-ketoglutarate (Goldinger and Barron, 1946) or succinate (Ball and Meyerhof, 1940; Ballentine, 1940; Goldinger and Barron, 1946) may be due in part to inactivation of the enzymes in egg extracts by echinochrome, and in part to failure of these substances to penetrate into intact eggs.

Homogenates capable of effecting oxidative phosphorylation have been prepared from unfertilized *Strongylocentrotus purpuratus* eggs by Lindberg and Ernster

(1948). Pyocyanine and DPN were added as electron carriers and glucose was added as a supplemental substrate.

f. Oxidation of other substrates. Ballentine (1940b) found, in Arbacia egg cytolysates, no evidence for oxidation of the following substrates: glucose, lactate, α-glycerophosphate, succinate, or butyrate.

Homogenates of other echinoderm eggs have been observed to oxidize a number of substrates in the presence of pyocyanine or other dyes as hydrogen acceptors (see Runnström, 1949, for review). In particular, Lindberg (1943) found that homogenates of Echinocardium eggs could catalyze the reduction of methylene blue by malic, formic, or phosphogluconic acids or by propanediol phosphate. The latter substance was studied because Lindberg isolated a phosphate fraction from sea urchin eggs corresponding in properties to propanediol phosphate. Lindberg (1943) has suggested that part of the energy for development of sea urchin eggs may be derived from oxidation of glucose-6-phosphate over the triphosphopyridine nucleotide-flavoprotein pathway (Warburg and Christian, 1936; Dickens, 1938).

g. Hydrolytic enzymes.

(1) *Adenosine triphosphatase and ATP.* This enzyme, which hydrolyzes adenosine triphosphate (ATP), has been found in eggs of *Strongylocentrotus purpuratus* (Connors and Scheer, 1947) and in *Paracentrotus lividus* (see Runnström, 1949); the ATP-ase activity of the fertilized Strongylocentrotus egg is reported to be higher than that for unfertilized, the amount of inorganic P liberated per hour per mg. egg nitrogen being 10.3 and 4.1 mg., respectively.

A substance similar to, or identical with, adenosine triphosphate was extracted from Arbacia eggs by White and Chambers (1949).

(2) *Phosphatase.* The acid phosphatase activity of the unfertilized Arbacia egg is 3–5 times that of the alkaline phosphatase, and remains constant during the first 40 hours of development; the alkaline phosphatase content remains constant until just before gastrulation and then rises to 10 times that of the unfertilized egg (Mazia, Blumenthal, Benson, 1948). Alkaline phosphatase activity toward the artificial substrate, glycerophosphate, was also studied at various stages of Arbacia development by Krugelis (1947).

(3) *Ribonuclease and desoxyribonuclease.* Evidence for ribonuclease activity in Arbacia eggs has been recorded by Bernstein (1949); Lansing and Rosenthal (1949) found some localization of ribonucleic acid at the surface of Arbacia eggs.

Mazia, Blumenthal, and Benson (1948) studied the distribution of desoxyribonuclease in the Arbacia egg. They found that in the unfertilized egg this enzyme is not restricted to nuclear structures, and that most of the desoxyribonuclease is in the cytoplasm, though no measurable amount of desoxyribonucleic acid is found there. During the first 40 hours of development after fertilization, there is no increase in the total desoxyribonuclease content of the egg, but the fraction of the enzyme which is non-sedimentable at 20,000 g. declines as development proceeds, being about 10–20 per cent of the total activity at 40 hours.

(4) *Peptidases.* Holter and co-workers (1936, 1938) studied the peptidase activity toward alanylglycine of unfertilized Arbacia eggs and fractions prepared from them by centrifuging. Holter found that the peptidase is localized in the hyaline ground substance (matrix) of the egg cytoplasm and not bound to granular matter.

Doyle (1938) found the peptidase content of *Psammechinus miliaris* to be the same before and after fertilization, in agreement with the experiments of Holter (1936) upon Echinarachnius.

Lundblad (1949) demonstrated proteolytic activity toward gelatin in eggs and sperm of *Arbacia lixula* and sperm of *Paracentrotus lividus.*

h. Catalase. Catalase activity was found in eggs and egg fragments of Psammechinus by Doyle (1938). Fertilized eggs with intact membranes showed lower catalase activities than unfertilized eggs. Fertilized eggs with membranes destroyed showed the same catalase activity as unfertilized eggs. (For earlier observations on catalase in Arbacia eggs, see Harvey review, 1932.)

2. METABOLIC INTERMEDIATES. During the first eight hours of development, the synthetic activities of the sea urchin egg consist chiefly in rearranging the nuclear and cytoplasmic components, with little or no increase in the total organic mass of the egg (Gray, 1927; E. B. Harvey, 1949). The question of what foodstuffs are available and used to provide energy for these synthetic activities according to the reactions illustrated in Figures 1–3 has been given only preliminary study in *Arbacia punctulata* eggs, and has received somewhat more attention in European sea urchins (see Örström and Lindberg, 1940; and Lindberg, 1946).

a. Total acid-hydrolyzable polysaccharide. This was reported to be equivalent to 50 mg. glucose per gram Arbacia egg protein by Perlzweig and Barron (1928), and 110 mg. glucose per gram egg protein by Hutchens, Keltch, Krahl, and Clowes (1942). The discrepancy was attributed by the latter authors, in part, to differences in the time of the season at which the eggs were collected and used, as they found that the carbohydrate values were higher from stored than from recently collected urchins. Confirming this suggestion, it was later found that the nitrogen content of the Arbacia egg tends to fall during the latter part of the season, especially if urchins are stored for several days or weeks in indoor aquaria (Crane, 1947). This nitrogen loss tends to elevate analytical values which are based on egg nitrogen or protein content.

Of the acid-hydrolyzable polysaccharide, only a negligible amount comes from the jelly (Hutchens, Keltch, Krahl, and Clowes, 1942).

No free reducing sugar was found by either group of investigators.

b. Glycogen. Of the 110 mg. total acid-hydrolyzable polysaccharide per gram egg protein found by Hutchens, Keltch, *et al.* (1942), 46 per cent was isolated as an alkali-stable, alcohol-precipitable polysaccharide. This substance corresponds to typical animal glycogen in optical rotation, yield of glucose upon hydrolysis, and reaction with iodine. This glycogen content is of the same order as that (50–80 mg. per gram egg protein) obtained for Arbacia by Blanchard (1935) and for the European sea urchins *Paracentrotus lividus* and *Echinus esculentus* (see Örström and Lindberg, 1940, for a summary of the total carbohydrate and glycogen contents of these eggs).

c. Other possible metabolic intermediates. Other metabolic intermediates indicated in Figures 1 and 2 have not been looked for in Arbacia eggs, but the phosphate fractions of European sea urchins have been studied by Zielinski (1939) and Lindberg (1943). In Echinocardium eggs, the latter reported that there are only three principal forms of phosphate; 53 per cent of the total being inorganic orthophosphate, 31 per cent adenosine triphosphate, and 16 per cent propanediolphosphate.

d. Fats. The total fat in 10^6 unfertilized eggs is 5.65 mg., of which 7.5 per cent is sterol and 38 per cent phospholipid. After fertilization, the total decreases up to 8 hours, increases to 20 hours, and then declines again up to 40 hours (Hayes, 1938).

Parpart (1941) found the total lipid to be 5.4 per cent of the whole egg, or 26.9 per cent of egg solids; 77 per cent of the lipid appeared to be bound to protein. Neither the total lipid nor lipid bound to protein was found to change during the first 5 hours of development.

Navez and E. B. Harvey (1935), Navez (1939), and Navez and DuBois (1940) have studied the unsaturated fatty acid fraction of Arbacia eggs, some component of which acts as a catalyst in oxidation of the Nadi reagent, thus simulating the enzyme indophenol oxidase.

e. Foodstuffs consumed and products formed during cleavage. Hutchens, Keltch, Krahl, and Clowes (1942) measured the glucose used, and the lactic acid, carbon dioxide, and ammonia formed by the fertilized Arbacia egg during the first few hours after fertilization. Their observations are as follows: (1) little or no lactic acid is formed; (2) the glucose used is, in 5 of 6 experiments, not adequate to account for the oxygen consumed; (3) the oxygen required for protein oxidation (calculated on basis of ammonia produced) is sometimes greater, sometimes less, than the oxygen consumption not accounted for by carbohydrate oxidation. For the periods in which the total oxygen consumption could not be accounted for by the sum of apparent carbohydrate and protein oxidation, the respiratory quotients are low, suggesting that fat may be oxidized.

Perlzweig and Barron (1928) had previously measured the lactic acid content before and immediately after fertilization.

Zielinski (1939) and Örström and Lindberg (1940) found a decrease in carbohydrate content of various sea urchins to occur upon fertilization, but they did not study the period 0.5–3 hours after fertilization, so that their results cannot be directly compared with those of Hutchens, Keltch, Krahl, and Clowes (1942).

3. PHOSPHORUS AND NUCLEIC ACID METABOLISM IN ARBACIA EGGS. Determinations of the total phosphorus of the Arbacia egg and its partition are given in Table II.

Measurements of the rate of uptake of inorganic P^{32} from sea water by Arbacia were made by Abelson (1947, 1948). He found the total P^{32} uptake by fertilized eggs in a given time to be 40 times that of the unfertilized eggs; the amount taken up by fertilized eggs at 10° C. was only one-seventh that at 23° C. Of the total P^{32} taken up by fertilized eggs, 97 per cent appeared in the acid-soluble fraction. Of this acid-soluble fraction, 16 per cent was inorganic phosphate, 41 per cent adenosine triphosphate and diphosphate, 41 per cent barium-soluble, and 2 per cent unaccounted for. These measurements were made using carriers for inorganic

TABLE II

Analyses of the phosphorus fractions of Arbacia eggs, milligrams P per gram wet weight egg or embryo (modified from Villee, Lowens, Gordon, Leonard and Rich, 1949)

Investigator	Stage	Acid soluble P	Phospholipid P	Acid insoluble P	DNA P	RNA P	Phosphoprotein P	Total P
Crane (1947)	unfert. eggs	1.3	1.3	0.86	0.03			3.2 [1]
Schmidt, Hecht, and Thannhauser (1948)	unfert. eggs	—	1.5	1.0	0.04	0.86	0.11	4.1
Villee *et al.* (1949)	3 hrs. embryos	1.4	—	0.86	0.05	0.74	0.06	—

[1] Analyses for individual fractions were carried out on separate batches of eggs, and the original values were based on egg nitrogen content. Later, Crane found that the nitrogen content of various batches of eggs decreased with increasing periods of storage of the urchins. For this reason, the total P is not equal to the sum of the individual fractions.

phosphate and ATP, and were recorded with some reserve because of interference of echinochrome with the phosphate fractionation.

The rates at which inorganic P^{32} enters the various organic fractions of the fertilized Arbacia egg were determined by Villee, Lowens, Gordon, Leonard, and Rich (1949). After 6 hours of development, the relative quantities of P^{32} in the various fractions were (expressed as counts per minute per mg. $P \times 10^3$): acid soluble—P, 20; RNA—P, 61; acid insoluble—P, 75; DNA—P, 109; phosphoprotein—P, 135. On the basis of the fact that P^{32} enters the DNA more rapidly than the RNA fraction, they conclude that the DNA of the fertilized egg is not formed from the RNA of the unfertilized egg, as had been suggested for eggs of *Paracentrotus lividus* by Brachet (1947).

The P^{32} uptake by other sea urchin eggs has also been measured. Lindberg (1949) has determined the rate at which P^{32} is incorporated into the acid-soluble, acid-labile fraction (presumed by Lindberg to be adenosine triphosphate) of Psammechinus eggs. In the unfertilized eggs, the initial rate of P^{32} uptake into this fraction is low, and a steady state is reached within 5 minutes after exposure to the P^{32}. In the fertilized egg, the rate of P^{32} incorporation into this fraction is much higher, and is sustained for at least 60 minutes after fertilization.

Measurements of P^{32} uptake have been made upon *Strongylocentrotus* (*purpuratus* and *franciscanus*) by Brooks and Chambers (1948), Chambers and White (1949), and upon *Lytechinus pictus* by Chambers, Whiteley, Chambers, and Brooks (1948).

Schmidt, Hecht, and Thannhauser (1948) reported that the unfertilized *Arbacia punctulata* egg contains 20×10^{-5} micrograms ribonucleic acid—P(RNA–P) and 1×10^{-5} micrograms desoxynucleic acid—P(DNA–P) per egg, i.e. 0.86 and 0.04 mg. per wet gram of eggs, respectively. During the first 24 hours of development, the amount of ribonucleic acid per embryo remains virtually unchanged, but the desoxynucleic acid steadily increases until, at the end of this period, the desoxyribosenucleic acid content per embryo is 10–15 times that of the unfertilized egg.

Blanchard (1935) had previously isolated 1.08 grams of a relatively pure sample of desoxynucleic acid from 4820 grams of wet unfertilized Arbacia eggs; he also isolated a small amount of ribosenucleic acid.

4. Calcium Metabolism. Heilbrunn, Mazia, and Steinbach (1934) and Mazia (1937) have found that the proportion of bound calcium ion in Arbacia eggs decreases by about 15 per cent on fertilization, the total calcium remaining constant. Mazia calculated that the free calcium of the unfertilized eggs is about 0.0005 molar, increasing by 0.001 molar upon fertilization. The relation of calcium ion to activation processes has been discussed by Heilbrunn (1943).

Changes in free calcium in other echinoderm eggs have been measured by Örström and Örström (1942) and Monroy-Oddo (1946). Hultin (1949) found that calcium ion increased both oxygen uptake and anaerobic acid formation in homogenates of Psammechinus eggs.

5. Echinochrome. The precise function of echinochrome in metabolism of the Arbacia egg has yet to be defined. The observations of Keltch and co-workers (1949, 1950), recorded above, suggest that echinochrome, when it leaves the pigment granules and gains access to the egg cytoplasm, may inactivate enzymes which contain sulfhydryl groups.

Crystalline echinochrome was isolated from the eggs and tests of *Arbacia punctulata* by Ball (1936). The pigment is capable of reversible oxidation-reduction and has a potential, E_0' at pH 7 and 30° C., of -0.221 V. and an E_0 of $+0.1995$ V. (Cannan, 1927). The structure of echinochrome from *Arbacia punctulata* eggs has not been determined, but that from *Arbacia lixula* eggs, which is presumed to be similar to that from *Arbacia punctulata,* has been shown to be (in the reduced form) 1,3,4,5,6,7,8-heptahydroxy-2-ethylnaphthalene (Kuhn and Wallenfels, 1939).

The spectrophotometric properties for echinochrome from the eggs are the same as for that from the amoebocyte and test of *Arbacia punctulata* (Ball and Cooper, 1949). Echinochrome has an acid group of pK′ 6.38 at 26° C. The acid form has 3 absorption peaks at 255, 335, 475 mμ, with molecular absorption coefficients of 1.93×10^4, 0.87×10^4, 0.65×10^4, respectively. The three peaks for the ionized form are centered at 275, 400, and 475 mμ with molecular absorption coefficients of 2.11×10^4, 0.88×10^4, and 1.12×10^4, respectively. The eggs contain on the average 0.58 gram echinochrome ($C_{12}H_{10}O_7$) per 100 cc. of eggs packed by centrifuging.

E. Penetration of Chemical Agents into Arbacia Eggs

The effects of many weak acids and bases upon Arbacia eggs are strongly dependent upon the pH of the sea water medium in which the eggs are suspended.

Detailed analyses of the relation of pH to intensity of physiological effect in Arbacia eggs have been made for carbon dioxide (Smith and Clowes, 1924; Haywood and Root, 1930, 1932); for substituted phenols (Krahl and Clowes, 1938; Hutchens, Krahl, and Clowes, 1939); for barbiturates (Krahl, 1940; Clowes, Keltch, and Krahl, 1940); and for local anesthetics (Krahl, Keltch, and Clowes, 1940).

The total concentration of each phenol, barbiturate, or local anesthetic required to produce 50 per cent inhibition of cleavage was measured at each of several extracellular pH values, and the concentration of undissociated molecules and ionized molecules was calculated in the conventional way from the equation:

$$\mathrm{pH} = \mathrm{pK}'_{\mathrm{a}} + \log \frac{\text{anion concentration}}{\text{undissociated molecule concentration}}$$

for the weak acids and the analogous equation:

$$\mathrm{pH} = \mathrm{pK}'_{\mathrm{water}} - \mathrm{pK}'_{\mathrm{b}} - \log \frac{\text{cation concentration}}{\text{undissociated molecule concentration}}$$

for bases, where $\mathrm{pK}'_{\mathrm{a}}$, $\mathrm{pK}'_{\mathrm{b}}$, $\mathrm{pK}'_{\mathrm{w}}$ are the negative logarithms of the dissociation constants of the acid, the base, or water, respectively. It was found, with any one agent, that the concentration of *undissociated molecules* required to produce 50 per cent inhibitition of the rate of cleavage is the same at various extracellular pH values, whereas neither the total concentration nor the ion concentration remains constant as the pH is varied. An example is shown in Table III. These observations were interpreted to indicate that these weak acids and bases penetrate Arbacia eggs principally, and in most cases entirely, in the form of undissociated molecules. In the course of these same studies, an attempt was made to identify the form in which these agents act within the cell.

TABLE III

Concentration of undissociated substituted phenol molecules required to produce 50 per cent reduction of the rate of cell division of fertilized eggs of Arbacia punctulata at pH 6.6 and pH 8.1 in glycyl glycine buffer (Krahl and Clowes, 1938a)

Temperature 20° C.

Substituted phenol	pK′	Total concentration		Extracellular molecule concentration	
		pH 6.6	pH 8.1	pH 6.6	pH 8.1
		moles per l.	moles per l.	moles per l.	moles per l.
2,4-dinitrophenol	4.1	3.4×10^{-6}	6.3×10^{-5}	1.1×10^{-8}	0.6×10^{-8}
4,6-dinitro-o-cresol	4.4	8.0×10^{-7}	1.5×10^{-5}	5.1×10^{-9}	3.8×10^{-9}
2,4,5-trichlorophenol	6.9	1.7×10^{-6}	1.5×10^{-5}	1.1×10^{-6}	1.1×10^{-6}
2,4-dichlorophenol	7.7	3.2×10^{-5}	1.1×10^{-4}	3.0×10^{-5}	3.1×10^{-5}
m-nitrophenol	8.3	1.7×10^{-4}	3.2×10^{-4}	1.7×10^{-4}	2.0×10^{-4}

Tyler and Horowitz (1937) found that the concentration of the anion of each of 8 substituted phenols required to produce 90 per cent inhibition of cleavage of eggs of *Strongylocentrotus purpuratus* is the same, from which they concluded that the active agent in blocking cleavage was the anion. In contrast, Krahl and Clowes (1938), using a more complete series of 30 substituted phenols, upon Arbacia eggs, found that the calculated anion concentrations required to produce 50 per cent inhibition of cleavage vary from $0.1 \times 10^{-6}\ M$ for 2,4-dinitro-o-cyclohexylphenol up to $192 \times 10^{-6}\ M$ for 2,4-dinitro-o-isopropylphenol. The measured values from which these calculations were made are given in Table IV. Thus, the attractive hypothesis of Tyler and Horowitz is not supported by more extensive data upon Arbacia. Krahl and Clowes (1938) and Hutchens, Krahl, and Clowes (1939), after studying effects of these agents at varying intracellular and extracellular pH values, drew the tentative conclusion that the undissociated molecule was the form active in inhibiting cleavage, while the anion might be the active agent in stimulating oxygen consumption (see below).

Though not studied in detail, the variation in effects of other weak acids with pH is qualitatively like that of the agents mentioned above. The physiological effects upon Arbacia eggs of hydrogen cyanide (pK′ 9.2), hydrazoic acid (pK′ 4.7), hydrogen sulfide (pK′ 7.0), and iodoacetic acid (pK′ 3.1) are more marked in acid

TABLE IV

Effects of substituted phenols on oxygen consumption and cleavage of fertilized eggs of Arbacia punctulata at pH 7.5 and 20° C.

(From Clowes and Krahl, 1936; Krahl and Clowes, 1936, 1938a)

Substituted phenol	pK′ at 25° C.	Oxygen consumption, maximum rate, as per cent of normal [1]	Cleavage, total conc. to give 50 per cent inhibition
			moles per l. × 10^6
1. 2,4-dinitro-o-cyclohexylphenol	4.2	—	0.6
2. 2,4-dinitro-ar-tetrahydro-α-naphthol	4.5	320	1
3. 4,6-dinitro-2-(1,1-dimethylhexyl)-phenol	—	260	1
4. 2,4-dinitrothymol	4.1	no stimulation	1
5. 4-nitrocarvacrol	4.2	—	2
6. 2,4,6-triiodophenol	6.6	180	3
7. 4,6-dinitro-o-cresol	4.4	300	4
8. 2,4-dinitro-α-naphthol	3.6	230	6
9. 2,4,5-trichlorophenol	6.9	250	10
10. 2,4-dinitro-o-phenylphenol	3.9	—	10
11. 2,4,6-tribromophenol	6.0	230	16
12. 2,4-dinitrophenol	4.1	290	23
13. 4,6-dichloro-o-cresol	7.7	190	25
14. 2,6-dibromo-4-nitrophenol	3.7	240	25
15. 4,6-dinitrocarvacrol	4.5	360	46
16. 2,6-dichloro-4-nitrophenol	3.7	230	55
17. 2,6-dinitrophenol	3.7	270	55
18. 4,6-dibromo-o-cresol	7.6	230	98
19. 2,4,6-trichlorophenol	6.2	220	98
20. 2,4-dichlorophenol	7.7	230	100
21. p-nitrophenol	7.2	325	100
22. 2-hydroxy-5-chlorobenzaldehyde	7.4	—	125
23. 2,4-dibromophenol	7.8	270	138
24. 2-nitro-4-chlorophenol	3.5	250	184
25. 2-hydroxy-5-bromobenzaldehyde	7.4	—	250
26. m-nitrophenol	8.3	230	314
27. 2,6-dinitro-4-chlorophenol	3.5	210	400
28. 2,6-dibromophenol	6.6	120	900
29. 2,4-dinitro-o-isopropylphenol	3.0	—	1024
30. 2,4,6-trinitrophenol	0.7	no stimulation	6900
31. o-nitrophenol	7.2	no stimulation	22,600

[1] The concentrations required to produce maximal stimulation of oxygen consumption are about 0.5-1 times those required to produce 50 per cent inhibition of cleavage.

than in neutral or alkaline solutions (Clowes and Krahl, 1940). The sodium or potassium salts of such weak acids dissolve to give alkaline solutions which must be neutralized and buffered in the physiological range.

These principles must be kept in mind in comparing measurements of various investigators who have, in many cases, taken insufficient account of the effect of pH upon penetration of the agent under study.

F. EFFECTS OF CHEMICAL AGENTS UPON OXYGEN CONSUMPTION, CLEAVAGE, AND METABOLIC ENZYMES

Effects of chemical agents upon Arbacia eggs have been studied from a number of points of view. The two principal objectives, with examples of agents used to approach them, are: First, elucidation of the processes by which energy is provided for development of the egg—low oxygen tension, cyanide, carbon monoxide, azide, sulfide, inhibitors of copper catalysts, iodoacetate, substituted phenols, urethane, naphthoquinones, and dyes; second, analysis of the biochemical mechanism of action of physiologically active agents, including mechanism by which agents penetrate cell surfaces—substituted phenols, barbiturates, local anesthetics, xanthines, penicillins, sulfonamides, colchicine, podophyllotoxin, and a number of other agents.

1. LOW OXYGEN TENSION. The problem of requirement of oxygen for cleavage of sea urchin eggs was first posed specifically by J. Loeb (1895); he found that Arbacia eggs could not undergo sustained cleavage when oxygen tension is reduced by replacement of air with hydrogen. More detailed study showed that cleavage is completely suppressed at a level of 0.4 per cent oxygen—99.6 per cent nitrogen, where the oxygen consumption is 20–30 per cent of normal (Amberson, 1928; Clowes and Krahl, 1940).

Cytological observations by E. B. Harvey (1930) and Fry (1937) indicate that detailed metabolic activities are blocked either immediately or within a few minutes after the eggs are brought under anaerobic conditions. This indicates that the Arbacia egg has neither a reserve energy source nor a mechanism for energy liberation upon which it can support cleavage when oxygen is lacking.

2. CYANIDE, CARBON MONOXIDE, AZIDE, SULFIDE, SUBSTANCES FORMING COMPLEXES WITH COPPER. The effects of each of these agents have been tested upon egg respiration and cleavage, and upon cytochrome oxidase from the eggs.

Cyanide—In the unfertilized egg, about 50 per cent of the oxygen consumption is cyanide sensitive (Clowes and Krahl, 1934; Robbie, 1946).

Effects of cyanide upon oxygen consumption and cleavage in fertilized Arbacia eggs have been measured by a number of investigators (Table V). For other data which are not in a form adaptable to tabulation see Brooks (1933, 1941), Brinley (1930), Blumenthal (1930), Barron and Hamburger (1932), Shapiro (1940), Krahl and Clowes (1940).

Loeb (1906) observed that fertilized *Strongylocentrotus purpuratus* eggs would not divide in solutions of potassium cyanide. Apparently the first simultaneous measurements on both respiration and cleavage in presence of cyanide were made by Warburg (1910) upon eggs of *Strongylocentrotus lividus*; cleavage was blocked at a concentration of 0.0001 M cyanide, in which oxygen consumption was reduced to about 20 per cent of normal. These observations by Warburg were the first in a long series which led eventually to his characterization of the *Atmungsferment* as a metalloprotein and to the isolation in pure form of the yellow enzymes (flavoproteins) and of triphosphopyridine nucleotide.

Cleavage of the fertilized Arbacia egg is blocked at 20° C. when oxygen consumption is reduced by cyanide to about 35 per cent of normal (Clowes and Krahl, 1940; Robbie, 1946), but the concentration required to produce this effect cannot be precisely stated, owing to the dependence of this concentration upon the conditions of measurement. Potassium cyanide, at pH values in the physiological range,

TABLE V

Effect of cyanide upon oxygen consumption and cleavage of sea urchin eggs

Investigator	Unfert. (U) or fert. (F)	Temp. °C.	pH of medium	Oxygen consumption			Cleavage, concentration required for 100 per cent inhibition
				Inhibition at cyanide conc. required for 100 per cent inhib. of cleavage	Degree of maximal inhibition	Conc. required for maximal inhibition	
				per cent of control	per cent of control	moles per l.	moles per l.
Warburg (1910)[1]	F	—	—	80	80	1×10^{-4}	1×10^{-4}
Loeb and Wasteneys (1911)[2]	F	20	—	75	—	—	4×10^{-5}
Loeb and Wasteneys (1913)[2]	F	14	—	67	—	—	3×10^{-5}
Barron and Hamburger (1932)[3]	F	24	8.2	—	100	1×10^{-3}	—
Clowes and Krahl (1934b)[3]	U	21	8.2	—	50	1.3×10^{-3}	—
Korr (1937)[3,4]	F	15	—	—	39	2.5×10^{-3}	—
		20	—	—	49	2.5×10^{-3}	—
		25	—	—	57	2.5×10^{-3}	—
Lindahl (1940)[5]	U	19–22	—	—	43	1×10^{-3}	—
	F	19–22	—	—	88	1×10^{-3}	—
Clowes and Krahl (1940)[3]	F	20	7.9	65	75	1×10^{-3}	1.6×10^{-4}
		24	7.9	—	84	1×10^{-3}	—
Robbie (1946)[3]	U	20	8.0	—	57	1×10^{-4}	—
	F	20	8.0	65	82	1×10^{-4}	1.5×10^{-5}

[1] *Strongylocentrotus lividus.*

[2] *Strongylocentrotus purpuratus.*

[3] *Arbacia punctulata.*

[4] Values are given for temperature from 11–26°. Only one cyanide concentration was used (2.5×10^{-3}M); this was stated to be higher than that required for maximum inhibition.

[5] *Paracentrotus.* Values are also given for oxygen tensions other than that in air.

is in equilibrium with HCN, which is volatile and distills into the alkali used to absorb CO_2 in respiratory measurements by the Warburg method. Robbie (1946) has given detailed procedures for minimizing this difficulty. The opposite situation obtains in the older measurements by the Winkler method where respiratory CO_2 was allowed to accumulate; here the pH tends to fall and make the required concentration of cyanide lower than it would be at pH 8. All methods agree in indicating that the critical concentration of cyanide to block cleavage of Arbacia eggs is about 10^{-5}–10^{-4} M in unbuffered sea water.

As the cyanide concentration is increased, the oxygen consumption passes through a minimum; at the maximally inhibiting cyanide concentration, the oxygen consumption of the fertilized eggs is reduced by some 75–80 per cent (Warburg, 1910; Clowes and Krahl, 1940; Robbie, 1946). The fact that Korr (1937) observed a smaller degree of inhibition (49 per cent at 20° C.) may be due to his use of a concentration of cyanide above that which gives maximum inhibition. Lindahl (1940) found the inhibition by 0.001 M cyanide to be greater at low oxygen tensions than at 100 per cent oxygen, and Robbie (1946) likewise found a greater inhibition by 10^{-4} M cyanide as oxygen tension was lowered.

Cytochrome oxidase activity of Arbacia eggs is strongly inhibited by low concentrations of sodium cyanide, 50 per cent at about 3×10^{-6} M, and 100 per cent at about 2×10^{-4} M under the conditions of these experiments (pH 6.9). The degree of inhibition by a given concentration of cyanide is not greatly altered by variation in concentration of cytochrome c over the range from 0.34×10^{-5} M to 1.36×10^{-5} M nor by use of p-phenylene diamine instead of hydroquinone as substrate (Krahl, Keltch, Neubeck, Clowes, 1941).

Carbon monoxide—Carbon monoxide has the specific property of entering into catalytically inactive light-reversible combination with the ferrous iron of certain iron-porphyrin compounds (Warburg, 1926; Keilin, 1930). Its effects on oxygen uptake by isolated tissues are considered at present to be entirely attributable to this mechanism; an inhibition by carbon monoxide is thus more strongly indicative of dependence of respiration upon iron-porphyrin compounds than inhibition by cyanide, azide, or sulfide, which can combine readily with other metal-containing pigments than ferric porphyrin compounds, and which can also act as reducing agents.

With $CO-O_2$ mixtures in the dark, cell division is approximately 50 per cent inhibited at 2 per cent oxygen—98 per cent CO; completely and reversibly inhibited at 0.7 per cent oxygen – 99.3 per cent CO, at which point oxygen consumption is about 30 per cent of that in the control (Clowes and Krahl, 1940). The complete cell division block occurs at the same level of oxygen consumption as when simple oxygen lack is the division inhibiting agency; hence carbon monoxide (in contrast to azide; see below) appears to have no effect on cleavage other than that arising from its effect on oxygen consumption.

Both the partial inhibition of oxygen consumption and the partial suppression of cleavage by mixtures of 1.6 per cent O_2—98.4 per cent CO, or 2.1 per cent O_2—97.9 per cent CO, are reversed by light (Clowes and Krahl, 1940).

Carbon monoxide produces a light-reversible inhibition of the cytochrome oxidase from Arbacia eggs (Krahl, Keltch, Neubeck, Clowes, 1941).

The inhibition constant, $K = \frac{n}{1-n} \cdot \frac{pCO}{pO_2}$, where n is the per cent inhibition in the dark and pCO and pO_2 are the respective carbon monoxide and oxygen partial pressures, was calculated. For the cell-free cytochrome oxidase system from Arbacia eggs the value is about 3.1–3.2, at either 0.34×10^{-5} *M* or 1.36×10^{-5} *M* cytochrome c and 0.02 *M* hydroquinone as oxidizable substrate; it is 4.5 at 1.36×10^{-5} *M* cytochrome c and 0.001 *M* hydroquinone. For the respiration of the intact fertilized Arbacia egg the value of this inhibition constant is about 60, as compared to 5 for yeast saturated with glucose as substrate (Warburg, 1927) and 21 for fertilized *Paracentrotus lividus* eggs (Runnström, 1930).

The light-reversible carbon monoxide inhibition of respiration and cleavage is perhaps the best, if not the sole, evidence for participation of an iron-porphyrin catalyst related to cytochrome oxidase in the sequence of oxidative reactions which yield energy to support cleavage. But the precise nature of this catalyst must be settled by further investigation, there being two anomalies to be explained: first, the very low concentration in the egg of cytochrome c, the usual substrate for the cytochrome oxidase system; and second, the greater apparent affinity of CO for cell-free cytochrome oxidase than for the CO sensitive enzyme of the intact egg. The CO inhibition constant may provide a valuable criterion for the identification of the substance which is actually oxidized by the cytochrome oxidase in the egg.

No inhibition of oxygen consumption of unfertilized Paracentrotus or Arbacia eggs by carbon monoxide can be demonstrated (Runnström, 1930; Brooks, 1941). Brooks (1941) reported that unfertilized Arbacia eggs consume carbon monoxide at the same rate, and fertilized eggs at one-third the rate, that they consume oxygen. Any such carbon monoxide consumption must be taken into account in a quantitative reexamination of the carbon monoxide inhibition constants, but it should be noted that correction for CO consumption would make the inhibition constant for the fertilized eggs even higher than that observed.

Azide—Only 50 per cent of the respiration of fertilized Arbacia eggs is insensitive to azide; moreover, 50 per cent inhibition of cleavage occurred at an azide concentration (3×10^{-3} *M* at pH 7.9) inhibiting respiration by only about 10 per cent; complete and reversible inhibition of cell division occurred at an azide concentration (5×10^{-3} *M*) inhibiting respiration by 50 per cent (Clowes and Krahl, 1940). When considered in conjunction with the effects of oxygen lack, cyanide, and carbon monoxide mentioned above, these measurements indicate that azide interferes with cleavage by a mechanism other than by simple reduction of oxygen consumption. A possible clue to the nature of this action is found in the observation that azide can block "high energy" phosphate transfer in a cell-free system (Loomis and Lipmann, 1949) and in yeast (Reiner and Spiegelman, 1947). Cytochrome oxidase of Arbacia is 50 per cent inhibited by 6.4×10^{-5} *M* azide at pH 6.8 and 20° C. (Krahl, Keltch, Neubeck, Clowes, 1941).

Sulfide—The effect of sodium sulfide on respiration and cell division of fertilized Arbacia eggs was determined in flasks of the Dixon and Keilin type, in which alkali is not present during the period of oxygen uptake, and distillation of volatile acids such as H_2S from the medium during the period of measurement does not occur. Cleavage is 50 per cent inhibited at a sulfide concentration (10^{-4} *M*) inhibiting respiration by only 10 per cent. Complete but not reversible inhibition of cleavage occurs at a sulfide concentration ($2 \times 10^{-4}\ M$) inhibiting respiration by about 50 per cent. Thus, the hydrogen sulfide-sulfide system, like the hydrazoic acid-azide system, appears to have some effect on cleavage which is not attributable merely to its effect on oxygen uptake (Krahl, Keltch, Neubeck, Clowes, 1941).

Cytochrome oxidase of Arbacia eggs is also inhibited by sodium sulfide, complete suppression of activity being produced at approximately $1 \times 10^{-3}\ M$ sodium sulfide at pH 6.9. But at higher concentrations, sulfide is rapidly oxidized by the egg cytochrome oxidase preparation, so that the precise inhibition-concentration relationships are difficult to establish.

Substances forming copper complexes—In view of the suggestion of Keilin and Hartree (1938) that cytochrome oxidase may be a copper compound, effects of a number of inhibitors of copper catalysts on egg respiration and cleavage and on Arbacia cytochrome oxidase were explored. Sodium diethyldithiocarbamate and 8-hydroxyquinoline did not inhibit either oxygen consumption of fertilized eggs or the cytochrome oxidase therefrom up to concentrations of $5 \times 10^{-3}\ M$. Potassium dithiooxalate ($10^{-3}\ M$), diphenylthiocarbazone ($10^{-4}\ M$), isonitrosoacetophenone ($2 \times 10^{-3}\ M$), or 8-hydroxyquinoline ($5 \times 10^{-5}\ M$) did not inhibit cleavage of the fertilized eggs. Diethyldithiocarbamate blocked cleavage irreversibly at a concentration of $3 \times 10^{-5}\ M$ (Table VIII); at higher concentrations, cleavage proceeded through the 16 cell stage, but the eggs were irreversibly injured, as they did not survive to swimming forms when returned to sea water at this point. This anomalous effect of the dithiocarbamates may be exerted by a mechanism other than combination with copper (Clowes and Krahl, 1940; Krahl, Keltch, Neubeck, Clowes, 1941).

3. SUBSTITUTED PHENOLS AND RELATED SUBSTANCES. The effects of these substances upon oxygen consumption and cleavage of Arbacia eggs have been intensively studied by Krahl, Clowes, Keltch, and their collaborators (1936, 1938, 1940, 1949). As the effects of these substances upon enzyme systems have proved to be relatively specific (see below), their effects upon Arbacia eggs may be given in some detail.

a. Effects on eggs. The typical effect is illustrated by the behavior of 4,6-dinitro-o-cresol. As the concentration is increased, oxygen consumption is increased to a maximum at a concentration of about $4 \times 10^{-6}\ M$. Above this concentration, oxygen consumption drops off, and a reversible complete block to cleavage sets in at about $2 \times 10^{-5}\ M$, at which point oxygen consumption is still above normal (Clowes and Krahl, 1934a, 1936). Ellis (1933) had previously noted that first cleavage of fertilized eggs of *Urechis caupo* was delayed by 0.001 per cent 2,4-dinitrophenol.

These effects are not dependent on reversible oxidation-reduction of the nitro groups, as halogenated phenols act like nitrophenols with respect to both respiration and cleavage of fertilized Arbacia eggs (Krahl and Clowes, 1936).

The concentrations required to stimulate respiration and to block cleavage of fertilized Arbacia eggs are summarized for 30 substituted phenols in Table IV. From these data it can be seen that these agents vary substantially in their ability to inhibit cleavage, whether total concentration, undissociated molecule concentration, or anion concentration be used as the index of effectiveness (Krahl and

Clowes, 1938). In general, the concentration required to produce 50 per cent inhibition of cleavage is equal to, or slightly larger than, that which produces optimum respiratory stimulation.

The active substituted phenols also stimulate oxygen consumption of unfertilized Arbacia eggs (Clowes and Krahl, 1934b, 1936) and of artificially fertilized eggs (Keltch and Clowes, 1947). In the latter case the optimum level of oxygen uptake attained is approximately the same as that produced by the same agent upon sperm-fertilized eggs; the cleavage of the artificially fertilized eggs is blocked at approximately the same concentrations of 4,6-dinitrocresol or 2,4,5-trichlorophenol as those required for sperm-fertilized eggs (Keltch, Walters and Clowes, 1947).

Stimulation of oxygen consumption of unfertilized eggs by substituted phenols has not resulted in artificial parthenogenesis, as measured by appearance of a fertilization membrane.

Inhibition by nitrophenols of sperm fertilization requires concentrations much higher than those which block cleavage (Clowes, Krahl and Keltch, 1937).

Observations upon related compounds which do not exhibit the typical substituted phenol effect upon respiration and cleavage of Arbacia are summarized by Clowes and Krahl (1936).

For combined actions of substituted phenols, carbon monoxide, and other agents, see Krahl and Clowes (1940).

Cytological studies by Fry (1935) showed that eggs first exposed to an active substituted phenol at the metaphase, anaphase, telophase, or resting stages apparently tended to cease development in the subsequent prophase. Fertilized eggs suitably exposed to cyanide or x-radiation also tended to accumulate in the subsequent prophase. At first sight, this suggested that all these agents had some especially intense action at prophase. To attempt to settle this point, Fry and Krahl (1936) constructed a mitotic time schedule which would indicate the proportion of eggs in all mitotic stages at various times after fertilization. This enabled estimates of the average *normal* time spent by any given egg in a given mitotic stage to be made, and showed that this time was much longer for prophase than for any other phase, as was well known by qualitative observations upon the living egg. When the results with substituted phenols were quantitatively compared with this mitotic time schedule, it was found that the time spent by the inhibited eggs in prophase was perhaps *slightly* greater (relative to normal) than for other phases, but that the accumulation of prophase figures was principally due to the long duration of prophase and the resultant greater chance of trapping eggs at this stage. These considerations should be kept in mind in postulating a specific effect of any agent upon a specific stage in mitosis.

A more detailed general discussion of the substituted phenols has been given by Clowes (1948).

b. Effects on enzymes. The mechanism of the two phases of the substituted phenol action on Arbacia eggs, namely, the stimulation of oxygen consumption on the one hand and the inhibition of cleavage on the other, has received intensive study.

The analysis of the first phase of the effect was long hampered by the fact that the oxidative stimulation was attainable only with intact cells. No augmentation of activity of any soluble dehydrogenase enzyme, flavoprotein, cytochrome, or cytochrome oxidase system by the substituted phenols has yet been found. The possibility that the substituted phenol anion might act like arsenate (Warburg and Christian, 1939) in substituting for phosphate in the triose phosphate dehydrogenase system was tested by Krahl (1947) with negative results. The current hypothesis is that the substituted phenols increase oxygen consumption by changing the relationship of one or more oxidative enzymes to the cell structure, in a way which gives a higher than normal overall rate of hydrogen (electron) transfer; the precise nature of this effect is not yet known (Krahl and Clowes, 1938b; Peiss and Field, 1948; Tepley, 1949). A foundation for further analysis of this oxidative stimulation has been laid by the observation that some stimulation of oxygen consumption by these agents can be obtained in cell-free granule systems from kidney (Loomis and Lipmann, 1948) and from Arbacia eggs (Crane and Keltch, 1949; Clowes, Keltch, Strittmatter, and Walters, 1949, 1950).

Efforts to account for the second phase of the substituted phenol effect, namely, the progressive inhibition of both oxygen consumption and cleavage in concentrations above the respiratory optimum, have revealed that these agents inhibit the following enzymatic activities: d-amino acid oxidase (Krahl, Keltch and Clowes, 1940), *Zwischen ferment* and cytochrome reductase (Haas, Harrer, and Hogness, 1942), phosphate accumulation and transfer by yeast cells (Hotchkiss, 1944; Spiegelman and Kamen, 1946). The concentrations required for inhibition in these systems were 10–100 times those required to inhibit cleavage of Arbacia eggs. Neither the cytochrome oxidase of Arbacia eggs (Krahl, Keltch, Neubeck, and Clowes, 1941) nor any water-soluble dehydrogenase is inhibited by physiologically active concentrations.

Observations which may be more pertinent to the supression of cleavage have just been reported by Clowes, Keltch, Strittmatter, and Walters (1949, 1950). They found that oxidative phosphorylation by a cell-free system from Arbacia eggs (see above) is inhibited by substituted phenols, in confirmation of the findings of Loomis and Lipmann (1948) upon kidney granules. The effects of the individual substituted phenols upon this cell-free phosphorylating system from the eggs are parallel to those of the same agents upon cleavage of the intact eggs in the following specific respects: (1) For each agent the block to both cleavage and phosphorylation is produced by concentrations just larger than that required for optimum oxygen consumption; (2) dinitrocarvacrol produces an increase in oxygen consumption in both the intact eggs and the particulate system, but blocks both cleavage and phosphorylation; on the other hand, its isomer, dinitrothymol, inhibits oxygen consumption in both systems and blocks both cleavage and phosphorylation; (3) p-nitrophenol stimulates oxygen consumption in both systems and blocks both cleavage and phosphorylation; its isomer, o-nitrophenol, is inactive toward oxygen consumption in both systems and blocks neither cleavage nor phosphorylation; (4) picric acid is completely inactive in both systems; (5) the concentrations of undissociated molecules required for each of five nitrophenols to block cleavage are exactly the same as those required to block phosphorylation.

That concentrations of 2,4-dinitrophenol ($5 \times 10^{-5}\ M$) which block phosphorylation in the cell-free system also block phosphorylation in the intact Arbacia egg was demonstrated by Abelson (1947) and by Villee, Lowens, Gordon, Leonard, and Rich (1949), who also found incorporation of P^{32} into all phosphate fractions to be inhibited; entrance of P^{32} into the DNA fraction being relatively most reduced.

Thus, there is reason to attribute the inhibition of cleavage to the interference with generation and transfer of "high energy" phosphate which the substituted phenols cause.

4. Naphthoquinones. The substance, 2-hydroxy-3(2-methyloctyl)-naphthoquinone-1,4 (SN 5949), prepared for test as an antimalarial, was found to have effects similar in some respects to those of the substituted phenols and in other respects to those of cyanide (Anfinsen, 1947). Below 7.4×10^{-8} *M*, respiration was stimulated; at 7.4×10^{-7} *M*, respiration of unfertilized and fertilized Arbacia eggs was inhibited by 50 per cent and 70 per cent, respectively; at the latter concentration cleavage was completely blocked.

Though the effects of this group of substances upon enzymes from eggs have not been studied, Ball, Anfinsen, and Cooper (1947) offer evidence that the naphthoquinones of this type may inhibit a respiratory step between cytochrome c and cytochrome b. It would be interesting to know whether echinochrome, the naphthoquinone pigment of the Arbacia egg, has similar effects.

5. Iodoacetate. Runnström (1935), using 0.03 *M* iodoacetate at pH values from 6.1 to 8.0, found that cleavage was abnormal and that the respiration of the fertilized eggs was strongly depressed; with maximal inhibition, the residual respiration is 40 per cent of the control rate. Addition of lactate or pyruvate plus the iodoacetate brings the respiration back nearly to normal, but the inhibition of cleavage is not overcome.

In sea water adjusted to pH 6.0 to permit penetration (see above), iodoacetic acid produces 50 per cent inhibition of cleavage at 1.7×10^{-3} *M* and 90 per cent inhibition at 3.4×10^{-3} *M*. At these concentrations, the rates of oxygen consumption are 67 and 54 per cent of the control values, respectively (Clowes and Krahl, 1940).

Ellis (1933) studied effects of iodoacetate and other reagents which combine with sulfhydryl groups upon cleavage of eggs of *Urechis caupo.*

Iodoacetate can combine with sulfhydryl groups and thereby inactivate enzymes which require this structure (Michaelis and Schubert, 1934; Hellerman, 1937; Barron and Singer, 1943). Its effects have not been studied on enzymes from the egg other than cytochrome oxidase, which it does not inhibit (Krahl, Keltch, Neubeck and Clowes, 1941).

6. Urethane and Related Carbamate Derivatives. Interest in this group of substances was initiated by the observations of Warburg (1910), who showed that 0.0005 *M* phenylurethane could block cleavage of Strongylocentrotus while reducing oxygen consumption by 20 per cent (at most). The effects of a number of other carbamate derivatives upon cleavage and oxygen consumption have now been studied (Table VIII). Cleavage in Arbacia was blocked by phenylurethane when oxygen consumption was reduced to 70 per cent of normal, the critical concentration being 0.001 *M* (Clowes and Krahl, 1940). Determinations of effects of carbamates upon cleavage of Arbacia, made prior to 1932, are summarized by Harvey (1932).

Urethane is one of the narcotic agents which Fisher and his co-workers have used in an effort to show whether or not the oxidative activities associated with cleavage (activity respiration) proceed via pathways different from the respiration of the unfertilized egg (resting respiration). They analyzed mathematically the relation between narcotic concentration, respiration, and cleavage and concluded that the activity respiration passes over a catalytic system which is either different from that operating in the resting egg or has a different affinity for the inhibitor. Observations consistent with the same conclusions were made upon chloral hydrate, azide, and sulfanilamide (Fisher and Henry, 1944; Fisher, Henry, and Low, 1944).

Cornman (1947) determined effects of various carbamates on cleavage of Lytechinus.

The enzymatic mechanism underlying the specific effect of the carbamates on cleavage remains to be defined, but is obviously worthy of study.

7. Dyes. After the demonstration by Harrop and Barron (1928) that dyes capable of oxidation-reduction could enhance oxygen consumption of red cells, the effects of dyes upon Arbacia eggs were measured from a number of points of view: to determine whether dyes could substitute for oxygen as hydrogen acceptors in presence of respiratory inhibitors (Brooks, 1941); to determine whether the excess oxygen uptake could be used to accelerate cleavage and development (Clowes and Krahl, 1936; Brooks, 1941; Shapiro, 1948a); and as an aid in specifying the mechanism by which oxygen transfer is carried out in the eggs (Barron, 1929, 1932; Runnström, 1935; Korr, 1937; Brooks, 1941).

The individual investigators agree that, in concentrations from 10^{-4}–10^{-3} M at pH 7–8, methylene blue, pyocyanine, and a number of other dyes can increase oxygen consumption of both unfertilized and fertilized Arbacia eggs, but they do not agree completely in regard to the effects of the same agents upon cleavage and developmental processes. Clowes and Krahl (1936), studying cleavage over the three hour period after fertilization, found all dyes tested to produce irreversible cleavage block at the optimum concentration for respiratory stimulation. Shapiro (1948a), studying time required to reach first cleavage, likewise found an inhibition which, however, was reversible. The difference between the results of Shapiro and those of Clowes and Krahl may well lie in the length of time the eggs were exposed to the reagent. Brooks (1941, 1943), studying effects of KCN, CO, and methylene blue upon unfertilized eggs, and the early cleavage, morula, blastula, gastrula, and pluteus stages, found 0.0001 M methylene blue to increase the rate of development during early cleavage stages and to increase the size of the pluteus of Arbacia. Shapiro (1948a) could not confirm this favorable effect upon cleavage.

Brooks (1947) reported artificial parthenogenesis of eggs of *Urechis caupo* and *Strongylocentrotus purpuratus* by oxidation-reduction dyes.

8. Barbiturates and Local Anesthetics. The effects of 30 barbituric acid derivatives (Clowes, Keltch, and Krahl, 1940) and of 16 local anesthetics (Krahl, Keltch, and Clowes, 1940a) upon cleavage of the Arbacia egg were determined in the course of a study of their mechanism of penetration into living cells. The results are given in Tables VI and VII.

With a representative barbiturate, 5-isoamyl-5-ethyl barbituric acid, the oxygen consumption was 75 per cent of control when division was inhibited 50 per cent and 20 per cent of control when division was inhibited completely. A typical local anesthetic, γ-(2-methylpiperidyl)-propyl benzoate, produced 64 per cent inhibition of cleavage while oxygen consumption remained at, or slightly above, normal. Thus, the local anesthetic bases, like azide, sulfide, and the carbamates, have specific inhibitory effects upon cleavage which are not dependent upon inhibition of oxygen consumption.

The actions of barbiturates and local anesthetics upon enzyme systems of the Arbacia egg have not been studied.

TABLE VI

Concentrations of substituted barbituric acids required to produce 50 per cent reduction of the rate of cell division of fertilized eggs of Arbacia punctulata at 20° C. and approximately pH 7.8–8.0[1]

(From Clowes, Keltch, and Krahl, 1940)

Substituted barbituric acid	pK′ at 20° for sea water	Final pH of medium	Total concentration for 50 per cent inhibition of cleavage
			moles per l. $\times 10^4$
1. 5-ethyl-5-(β-ethylhexyl)	7.75	8.00	2
2. 5-allyl-5-(α-methylbutyl)	7.92	7.81	2
3. 5-benzyl-5-isopropyl	7.82	7.97	4
4. 5-(β-bromoallyl)-5-(α-ethylpropyl)	7.58	8.00	5
5. 5-ethyl-5-(α-methylbutenyl-2)	7.78	7.99	5
6. 5-(β-methylallyl)-5-(α-methylbutyl)	7.80	7.86	7
7. 5-ethyl-5-hexyl	7.66	8.00	8
8. 5-ethyl-5-(α-methylbutyl)	8.02	8.00	12
9. 5-(β-bromoallyl)-5-(α-methylpropyl)	7.60	8.00	13
10. 5-ethyl-5-(α-ethylpropyl)	7.89	7.98	14
11. 5-allyl-5-benzyl	7.06	7.92	15
12. 5-amyl-5-ethyl	7.82	7.93	15
13. 5-(α, γ-dimethylbutyl)-5-ethyl	7.99	7.90	15
14. 5-isoamyl-5-ethyl	7.78	8.04	15
15. 5-cyclopentenyl-5-ethyl	7.98	7.97	17
16. 5-ethyl-5-(β-phenylethyl)	7.74	7.99	22
17. 5-allyl-5-isobutyl	7.54	8.00	39
18. 5-isobutyl-5-(β-methylallyl)	7.62	7.93	40
19. 5-butyl-5-ethyl	7.76	7.97	40
20. 5-sec-butyl-5-crotyl[2]	—	8.03	42
21. 5-(β-bromoallyl)-5-isopropyl	7.59	8.00	48
22. 5-ethyl-5-cyclohexenyl	7.36	8.00	58
23. 5-ethyl-5-phenyl	7.26	7.92	95
24. 5-allyl-5-isopropyl	7.73	7.97	98
25. 5-cyclohexenyl-1,5-dimethyl	8.24	8.74	104
26. 5,5-diallyl	7.62	7.96	160
27. 5-ethyl-5-isopropyl	7.90	8.02	162
28. 5-ethyl-5-piperidyl	7.56	7.92	200
29. 5,5-diethyl	7.75	7.94	320

[1] The concentrations required at pH 6.4–6.7 and at pH 8.6–8.9 were also determined.

[2] Derivative of thiobarbituric acid rather than of barbituric acid itself.

9. MISCELLANEOUS. The effects of many other agents, with unknown effects upon metabolic enzymes, have been tested upon respiration and cleavage of Arbacia eggs.

Since these do not contribute directly to the principal objective of this paper, namely, to elucidate the metabolic processes by which energy is provided by cleavage, they will not be dealt with here in detail. But all the miscellaneous agents which have been employed in the period 1932–1949 are listed below, and quantitative data for them, where available, are given in Tables VIII and IX.

TABLE VII

Concentrations of local anesthetics required to produce 50 per cent reduction of the rate of cell division of fertilized eggs of Arbacia punctulata at 20° C. and approximately pH 7.8–8.0[1]

(From Krahl, Keltch, and Clowes, 1940)

Local anesthetic	pK′ at 20° for sea water	Final pH of medium	Total concentration for 50 per cent inhibition of cleavage
			moles per l. × 10^4
1. N,N-Diethyl-N′-(2-butyloxy-quinoline-4-carboxyl)-ethylenediamine ('Dibucaine')	5.03	7.99	0.06
2. α-Piperidyl-β,γ-dicarbanilido-propanediol ('Diothane')	5.84	7.94	0.3
3. β-Dimethylaminoethyl-p-butylaminobenzoate ('Tetracaine')	5.50	7.98	0.9
4. 4,4′-Diethoxy-N,N′-diphenyl-amidine ('Phenacaine')	4.78	7.90	1.3
5. 2-Benzoxy-2-methyl-1-dimethylaminobutane ('Stovaine')	5.42	7.90	1.3
6. γ-Dibutylaminopropyl-p-aminobenzoate ('Butacaine')	4.90	7.91	3.1
7. 4-Benzoxy-2,2,6-trimethylpiperidine ('β-Eucaine')	4.27	7.90	4.5
8. γ-Diethylaminopropylcinnamate ('Apothesine')	4.06	7.93	5.2
9. β-Diethylaminoisohexyl-p-aminobenzoate ('Panthesine')	4.44	7.90	7.0
10. 2-Benzoxy-2-dimethylaminomethyl-1-dimethylaminobutane ('Alypin')	4.55	7.92	7.2
11. N-Methyl-l-benzoylecgonine ('Cocaine')	4.97	7.78	10
12. β,β-Dimethyl-γ-diethylamino-propylbenzoate ('Larocaine')	4.21	7.91	10
13. γ-(2-Methylpiperidyl)-propylbenzoate ('Metycaine')	4.18	8.00	13
14. Ethyl-p-aminobenzoate ('Benzocaine')	10.82	7.97	15
15. β-Diethylaminoethyl-p-aminobenzoate ('Procaine')	4.73	7.93	23
16. 2-(p-Aminobenzoxy)-2-methyl-1-dimethylaminobutane ('Tutocaine')	4.82	7.93	45

[1] The concentrations required at pH 6.4–6.7 and at pH 8.6–8.9 were also determined.

Acetyl salicylic acid—Budington (1935)

Acids and bases—Clowes, Keltch, and Wade (1933, 1934); Keltch, Wade, and Clowes (1933)

Amyl alcohol—Clowes and Keltch (1941)

Caffeine and related compounds—Cheney (1945, 1948)

TABLE VIII

Effect of various substances upon oxygen consumption and cleavage of fertilized Arbacia eggs

Agent	T° C.	pH	Oxygen consumption			Cleavage		
			Maximal change produced	Conc. required to produce maximal change	Change at conc. required to inhibit cleavage maximally	Maximal inhibition	Conc. required to produce maximal inhibition	Reversibility of maximum inhibition
			per cent of control	moles per l. × 10^{4}	per cent of control	per cent of control	moles per l. × 10^{5}	
Miscellaneous								
n-Amylalcohol[1]	20	8.0				100	660	none
Caffeine[2]	25	7.9	−60	250	−60	100	250	not stated
Carbon dioxide[3]	21		−80		−70	—	—	not stated
Chloral hydrate[6]	24	7.3–8.3	−80	1000	−60	100	63	partial
Chloroform[1]	20	8.0				100	160	none
Colchicine[4]						100	1	not stated
Colchicine[5]						100	2	not stated
Ethyl carbamate[6]	24	7.3–8.3	−75	6300	−30	100	1400	yes
Hydroxyethylapocupreine[1]	20	7.8				100	41	none
p-(α-Methylbutylamino) benzoate[1]	20	7.8				100	10	partial
Penicillin[7] (non-crystalline)	20	7.5				100	5000[7]	not stated
Phenylmercury nitrate[1]	20	6.9–8.6				100	0.02	none
Podophyllotoxin[8]						100	0.01	not stated
Tetrachlorohydroquinone[1]	20	7.9				100	0.6	none
Tetrachloroquinone[1]	20	7.9				100	1	none
Usnic acid[9]						100	3	not stated
Sulfonamides and related compounds								
n-Butylsulfone-p-phenetidide[1]	20	7.9				65	20	yes
Diaminodiphenylmethane[1]	20	7.9				100	50	yes
Diaminodiphenylsulfone[1]	20	7.9	−43	20	−35	60	9	yes
Ethylsulfone-p-phenetidide[1]	20	7.9				100	16	yes
Methylsulfone-p-phenetidide[1]	20	7.9				40	41	yes
p-Nitrosobenzenesulfonamide[1]	20	7.9			0	100	2	none
n-Propylsulfone-p-toluidide	20	7.9				100	20	yes
Sulfanilamide[10]	25	8.0	−48	400	−45	100	400	yes
Sulfanilylethanolamine[1]	20	7.9				60	280	none
N^4-Trichloroacetyl-sulfanilamide[1]	20	7.9				70	20	none

TABLE VIII—*Continued*

Agent	T° C.	pH	Oxygen consumption			Cleavage		
			Maximal change produced	Conc. required to produce maximal change	Change at conc. required to inhibit cleavage maximally	Maximal inhibition	Conc. required to produce maximal inhibition	Reversibility of maximum inhibition
			per cent of control	moles per l. × 10^4	per cent of control	per cent of control	moles per l. × 10^4	
Dyes								
o-Cresol indophenol [12]	20	7.9	+75	3	+30	100	1	none
Dimethyl-p-phenylene diamine [12]	20	7.9	+125	10	+120	100	2	none
Methylene blue [12]	20	7.9	+60	10	+50	100	5	none
Neutral red [12]	20	7.9	+25	1	0	90	10	none
Pyocyanine [12]	20	7.9	+200	5	+90	100	30	none
Pyocyanine [11]	—	—	+96	5	—			
Tetramethyl-p-phenylene diamine [12]	20	7.9	+250	10	+250	100	10	none
Dithiocarbamates								
sec-Butyl ethyldithiocarbamate [1]	20	7.9				80[13]	0.3	none
Di-n-butyldithiocarbamate [1]	20	7.9				100	41	none
Diethyldithiocarbamate [1]	20	7.9	+40	1	0	85[13]	0.3	none
Di-isoamyldithiocarbamate [1]	20	7.9				100	5	none
Dimethyldithiocarbamate [1]	20	7.9				50	81	none
Di-n-propyldithiocarbamate [1]	20	7.9				100	0.3	none
N,N′-Dimethyl-(2,4-dinitrophenyl)-dithiocarbamate [1]	20	7.9				100	20	none
Isoamylethyldithiocarbamate [1]	20	7.9				100[13]	0.2	none
Isobutylethyldithiocarbamate [1]	20	7.9				100[13]	0.3	none

[1] Clowes and Keltch (1939, 1940, 1941).

[2] Cheney (1945, 1948).

[3] Root (1930). The maximal effect on oxygen consumption was measured at 180 mm. CO_2, 0.0018 M $NaHCO_3$, pH 5.50. The maximal effect on cleavage as measured by the cleavage delay method was obtained at 40 mm. CO_2, 0.0018 M $NaHCO_3$, pH 6.15, at which point oxygen consumption was reduced by 70 per cent.

[4] Nebel (1937).

[5] Beams and Evans (1939).

[6] Fisher and Henry (1944).

[7] Henry and Henry (1945). The concentration is stated in Oxford units per ml.

[8] Cornman (1947).

[9] Marshak and Harting (1948).

[10] Fisher, Henry, Low (1944).

[11] Runnström (1935b). Pyocyanine at 5 × 10^{-4} M also increased oxygen consumption of unfertilized eggs by 210 per cent.

[12] Clowes and Krahl (1936).

[13] A smaller degree of inhibition was produced by higher concentrations of this substance. This effect, while reproducible, is not understood.

TABLE IX

Delay in first cleavage of fertilized Arbacia eggs produced by various chemical agents

Agent	Concentration	T° C.	pH	Delay in first cleavage
				minutes
CO_2-bicarbonate[1]	10 mm. CO_2-0.0018 M $NaHCO_3$	20	6.76	16
	40 mm. CO_2-0.0073 M $NaHCO_3$		6.70	44
	80 mm. CO_2-0.0143 M $NaHCO_3$		6.67	108
	20 mm. CO_2-0.0018 M $NaHCO_3$		6.41	30
	80 mm. CO_2-0.0073 M $NaHCO_3$		6.41	260
Methylene blue[2]	1×10^{-4} M	26		2
	2×10^{-4} M			5
	4×10^{-4} M			10
Toluylene blue[2]	4×10^{-4} M	26		57
Ascorbic acid[3]	4×10^{-5} M	26.1		6
	2×10^{-4} M			7
	9×10^{-4} M			16
	2×10^{-3} M			30

[1] Haywood and Root (1932).
[2] Shapiro (1948a).
[3] Shapiro (1948b).

Carbon dioxide—Smith and Clowes (1924); Root (1930); Haywood and Root (1930, 1932)

Carcinogenic hydrocarbons—Keltch, Krahl, and Clowes (1937)

Chloral hydrate—Fisher and Henry (1944)

Chloroform—Clowes and Keltch (1941)

Colchicine—This substance is said to stop mitosis at metaphase, Nebel (1937); Beams and Evans (1940)

Dithiocarbamates—Clowes and Keltch (1941)

Ethyl alcohol—Waterman (1936)

Hydroxyethylapocupreine—Clowes and Keltch (1940)

Hypotonic sea water—Cornman (1943)

Leukotaxine—Menkin (1940)

Malonic acid—Rulon (1948)

Malononitrile—This substance interferes with phosphate transfer in intact eggs, Villee, Lowens, Gordon, Leonard and Rich (1949)

Metal ions—Alkali metals (Li, Na, K)—Chambers and Chambers (1938); Runnström (1935c); Moore, Bliss, and Anderson (1945); *copper*—Glaser (1924); Finkel, Allee, and Garner (1942); Runnström (1939); *other heavy metals*—Page (1929); Waterman (1937)

Nitrogen mustards—Agents of this group have been studied by Barron, Seegmiller, Mendes, and Narahara (1948) and by Hutchens and Podolsky (1948). No inhibition of oxygen consumption of the eggs was observed. Hutchens and Podolsky found the cleavage over a 3 hour period to be reduced to 75 per cent of control by tris-(β-chloroethyl)amine at $3 \times 10^{-5}\ M$ and by various bis-(β-chloroethyl) amines at $2 \times 10^{-4}\ M$.

Penicillins—Non-crystalline samples of penicillin had a slight inhibitory effect on cleavage at concentrations several thousand times those required to stop growth of sensitive bacteria (Henry and Henry, 1945), but highly purified samples of various penicillins were virtually without effect upon either respiration or cleavage (Clowes and Keltch, 1946).

Phenylmercury nitrate—Clowes and Keltch (1940)

Podophyllotoxin—Cornman (1947)

Pressure—Marsland (1939)

Rhodamine B—Clark (1940)

Sulfhydryl-blocking agents—Barron, Nelson, and Ardao (1948); Clowes and Keltch (1940)

Sulfonamides—Fisher, Henry, and Low (1944); Clowes and Keltch (1939)

Temperature—Rubenstein and Gerard (1933); Hoadley and Brill (1937); Tyler and Humason (1937); Borei (1949); Borei and Lybing (1949)

Tetrachlorohydroquinone—Clowes and Keltch (1940)

Ultraviolet light—Giese (1938a, 1938b, 1939); Harvey and Hollaender (1938)

Uranyl nitrate—Effects of this substance upon egg respiration have not been reported, but it inhibits respiration of Arbacia sperm (Benedict and Barron, 1946). It also inhibits phosphate transfer in the intact egg (Villee, Lowens, Gordon, Leonard, and Rich, 1949).

Usnic acid—This substance inhibits cleavage and phosphate transfer (Marshak and Harting, 1948), and also desoxyribonuclease (Marshak, 1949).

Vitamin C—Shapiro (1948b)

X-rays—Henshaw (1932, 1938, 1939); Henshaw and Francis (1936); Cohen (1939); Evans (1940); Evans and Beams (1939)

The following agents have been found to be without effect upon cleavage of fertilized Arbacia eggs when tested at the molar concentrations shown, which were the highest concentrations employed (Clowes and Keltch, 1939–1941): diethylmethylcarbinylurea, 8×10^{-3}; 4,4′-dinitrobenzophenone, 6×10^{-4}; 2,4-dinitrophenylmercaptobenzothiazole, 2×10^{-3}; morphine, 2×10^{-2}; phenacetine, 8×10^{-3}; porphyrexide, 1×10^{-3}; 2-sulfanilylaminothiazole, 5×10^{-4}; N-sulfanilylbenzamide, 5×10^{-4}; sulfapyridine, 8×10^{-3}; synthalin, 2×10^{-3}.

G. SUMMARY

1. This review deals with the general question of relation of metabolic activities to cleavage in the Arbacia egg. According to current biochemical concepts the answer to this question would be found in complete information about the following subjects:

a. The identity and interrelationships of the catalysts by which the eggs derive energy from foodstuffs.

b. The reactions by which energy is transferred to make possible the synthesis of structural and functional elements of the egg, such as carbohydrates, proteins, lipids, coenzymes, nucleic acids and other substances, many of which may yet be unknown.

c. The chemical and physical chemical rearrangements of these structural functional elements which lead to the biological event of cleavage.

The third subject is completely beyond investigation by present techniques, and the second is just being opened to investigation in the living egg by application of isotopic labelling techniques. This review is concerned, therefore, chiefly with the nature and interrelationships of the catalysts by which Arbacia eggs derive energy from foodstuffs.

2. The metabolic catalysts which have been shown to be *available* in the Arbacia egg for deriving energy from foodstuffs are:

a. An enzyme with some, but not all, of the properties of the cytochrome oxidase of other animal tissues. After extraction from the egg it oxidizes cytochrome c, but whether this activity is related to its biological function in the egg is an open question, as no biochemically significant quantity of cytochrome c has yet been conclusively demonstrated in the eggs, although it is present in the sperm.

The enzyme is poisoned by carbon monoxide, cyanide, azide, and sulfide, but not by copper-binding reagents. Its relative affinities for oxygen and carbon monoxide are, in the cell-free state, the same as those for cytochrome oxidase of mammalian muscle but, in the intact Arbacia egg, these relative affinities are intermediate between those of the usual cytochrome oxidase and those of other iron-porphyrin compounds such as hemoglobin.

b. The coenzymes, flavin-adenine dinucleotide, diphosphopyridine nucleotide, and diphosphothiamine. The properties of the enzymes of which these coenzymes are parts have not been investigated.

c. A mechanism for oxidative disposal of pyruvate.

d. An enzyme complex which can oxidize oxalacetate, α-ketoglutarate, or succinate with concomitant disappearance of inorganic phosphate, presumably via generation of "high energy" phosphate bonds and esterification of suitable phosphate acceptors.

e. The following enzymes which have undefined roles with respect to energy production and to chemical syntheses in the egg: dehydrogenases for various substrates other than those already mentioned; phosphatases; ribonuclease; desoxyribonuclease; catalase; peptidases.

The distribution of various enzymes in the egg has been studied with very suggestive findings. In the unfertilized or recently fertilized egg the total reducing activity, cytochrome oxidase, and desoxyribonuclease are quantitatively most abundant in the cytoplasmic matrix, i.e. in solution or upon the small granules less than 1 μ in diameter. As development proceeds, a progressively greater fraction of the enzymes are associated with larger granules of the type which stain as mitochondria. In view of these observations and the fact that the capacity for oxidative phosphorylation is found, in the adult mammalian tissues so far studied, to be associated with mitochondrial particles, the suggestion arises that localization of enzymes on specific particles may parallel development of specific function.

3. What is the *actual* pathway connecting enzyme activities with cleavage? Such answers to this question as are available come largely from experiments designed to block a specific metabolic enzyme system and to observe the degree to

which the egg can or cannot cleave when deprived of this enzyme or group of enzymes.

A note of caution should be emphasized here. The problem of explaining physiological effects in chemical terms is an exceedingly difficult one. It involves the demonstration not only that a given effect of a reagent upon an enzyme is *sufficient* to explain the physiological effect, but also the demonstration that the chemical effect offered as explanation is the only one possible, i.e. is a *necessary* explanation. Only the sufficiency criterion is usually met. In view of these considerations it is clear that conclusions about the relation of enzymes to function, which are based on action of inhibitors, must be only provisional. With this reservation in mind, the main findings about enzymatic activities and cleavage may be summarized.

a. For energy to support cleavage, eggs of Arbacia are completely dependent on aerobic processes. Like other animal cells, the eggs can form lactic acid but this process cannot support cleavage.

The nature of the foodstuffs oxidized is not settled; evidence for oxidation of carbohydrate or its phosphorylated breakdown products, of fats, or of amino acids has been offered, but is not conclusive.

b. Evidence for participation of two enzyme systems in oxidative energy-yielding reactions has been presented.

(1) *The cytochrome oxidase system.* The principal indication for participation of this oxidative step is that both the inhibition of oxygen consumption and the inhibition of cleavage by carbon monoxide are *light reversible.* Cytochrome oxidase is the only metabolic catalyst known to be subject to light-reversible inhibition by carbon monoxide.

(2) *An oxidative phosphorylation system.* The principal evidence is the highly specific parallel between inhibition of cleavage and inhibition of a cell-free phosphorylating system from the eggs by a series of substituted phenols. Supporting evidence is found in the fact that these agents likewise block phosphorylation and phosphate transfer in the intact fertilized egg.

c. In an effort to reveal the metabolic steps essential for cleavage, comparisons of the metabolic activities of unfertilized and fertilized eggs have been made.

The oxygen consumption of intact Arbacia eggs increases upon fertilization to a degree which depends on the temperature, by a factor of about 10 times at 11° C., by a factor of about 2 times at 30° C., and negligibly at 32° C. The higher respiration of the fertilized egg is relatively more sensitive than that of the unfertilized both to inhibitors of cytochrome oxidase, such as cyanide and carbon monoxide, and to inhibitors which act at other loci, such as narcotics.

The concentration of cytochrome oxidase is the same in unfertilized and fertilized eggs, and in great excess relative to oxygen consumption of intact eggs. The concentration of total dehydrogenase activity, as measured by ability to reduce ferricyanide, is likewise unchanged upon fertilization.

The ability to dispose of pyruvate, on the other hand, appears to be increased following fertilization.

From these and related facts, all observers appear to agree that the increase in oxygen consumption upon fertilization is the consequence of the introduction of a new or accelerated step between some substrate, perhaps pyruvate, and cytochrome oxidase. The nature of the new or accelerated step, a frequent subject of speculation, is unknown. It is not known to what extent, if any, the energy liberated by the excess oxygen consumption is used for synthetic processes and for cleavage.

The precise mechanism by which oxidative energy is made available for cleavage remains to be defined.

4. In the interest of completeness, and to provide a foundation for the further study of relation of metabolic activities and cleavage, the effects of all agents so far tested upon oxygen consumption and cleavage of Arbacia eggs have been recorded, even though their effects upon specific enzymatic activities frequently still await investigation. Examples of the viewpoints from which these substances have been investigated, and of the agents employed, are:

a. To demonstrate participation of various enzyme systems in yielding energy for cleavage: poisons for cytochrome oxidase such as cyanide, carbon monoxide, azide, sulfide; poisons for phosphorylation processes such as substituted phenols, azide, usnic acid; and poisons for other metabolic enzymes such as naphthoquinones and iodoacetate.

b. To learn more about the mechanisms by which physiologically active substances affect growth and cell division: acids and bases, carbamates, colchicine, nitrogen mustards, sulfonamides, penicillin.

c. To determine the form in which toxic ionizable substances penetrate living cells such as Arbacia eggs: substituted phenols, barbiturates, local anesthetics, other weak acids and bases. These agents appear to gain access to the interior of the Arbacia egg chiefly, if not entirely, as undissociated molecules.

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STUDIES ON THE PHYSIOLOGY OF UCA RED CHROMATOPHORES [1]

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Red chromatophores are present in considerable abundance in most individuals of *Uca pugilator* that are collected in the salt marshes in and near Woods Hole, Massachusetts. The abundance is sufficient to provide the animals with a distinctly reddish tint. Almost nothing has been reported concerning the responses of these chromatophores to background or to other factors, nor have any systematic studies been made as to the nature of the control of these chromatophores within the organism. Carlson (1936) reported that the red pigment was concentrated in eyestalkless *Uca pugilator*. This was confirmed by Abramowitz (1937) who reported further that injection of eyestalk extract into eyestalkless specimens caused red dispersion.

In view of the rapidly increasing information on the physiology of the black and white chromatophores of the fiddler crabs, and the various sources of chromatophorotropins involved, it seemed wise at this time to examine the red chromatophores, comparing them with the other chromatophores in their responses, to learn to what extent they were governed by the same or similar mechanisms, and to what extent by quite different ones.

Responses to Black and White Backgrounds

A number of experiments were performed to determine the character and magnitude of changes in the state of red chromatophores in response to black and white backgrounds. In a typical experiment, twenty fiddler crabs were divided into two lots of ten each; one lot was placed in a black enamelled container, the second in a white one. After about three hours in these situations the average state of dispersion of the pigment of the red chromatophores was recorded, using the well-known chromatophore index in which a chromatophore with its pigment in punctate condition is designated as 1, and one with its pigment completely dispersed, as 5, with 2, 3, and 4 describing three intermediate states. The backgrounds of the two lots of animals were then interchanged and the average state of the red pigment in the two lots determined after one, two, and three hours. The results of this experiment, conducted at an illumination of 100 f.c., are shown in Figure 1. It is readily seen that the pigment responds extensively, in fact through almost its entire normal range of change, during the daytime, at constant incident illumination, and in response simply to these changes in albedo.

In the foregoing characteristic, the red chromatophores of *Uca pugilator* differ quantitatively in their responses from the black and white chromatophores, whose responses to albedo, when incident illumination, temperature, and time in the daily

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rhythmical cycle of color change are constant, are of relatively small magnitude (Brown and Sandeen, 1948).

The red chromatophores of *Uca,* therefore, appear in their responses well adapted for alteration of the shade of the body to accord more closely with that of the background upon which the animal is located.

DIURNAL RHYTHM OF RED CHROMATOPHORE STATE

In conditions of constant darkness, the red chromatophores of *Uca pugilator* exhibit a very striking persistent rhythmicity of activity. This rhythm is illustrated in Figure 2A (solid line) in which it is readily observed that the pigment is more or less broadly dispersed by day and almost completely concentrated by night. The darkened blocks immediately above and below the plotted rhythm indicate the hours 6 P.M. to 6 A.M. The bottom band in the figure indicates the experimental illumination which, in this instance, is constant darkness. The amplitude of the rhythm was, in fact, substantially greater in this case than that of the rhythm simultaneously occurring in the black chromatophores of these animals (broken line in Figure 2A). The two rhythms are, however, clearly seen to be in phase with one another.

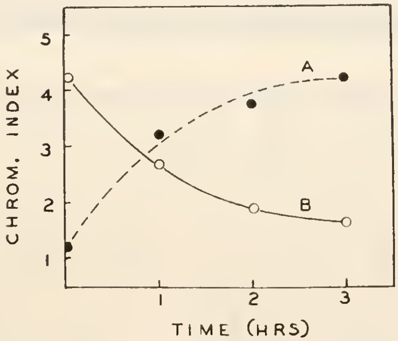

FIGURE 1. Changes in the red chromatophores in response to change of background: A. White to black; B. Black to white.

The rhythm of the red chromatophores was observed to persist with no loss in amplitude, and completely synchronized with the solar day-night cycle for four days.

In an attempt to demonstrate whether the red and black chromatophores were able, in a normal animal, to show activities in opposite directions to one another at any given moment, ten animals were exposed to twelve hours of illumination by night and to twelve hours of darkness by day over a period of three days. They were maintained during the experiment upon a white enamelled background. The results of this experiment are shown in Figure 2B between points *a* and *b*. The red chromatophores (solid line) showed dispersion of their pigment during the darkened day-time period, and concentrated their pigment during the illuminated night-time period in response to the white background.

The black chromatophores (broken line), on the other hand, governed predominantly by the endogenous persistent rhythmicity, altered the phase of their rhythm in response to the reversed condition of periods of illumination in such a manner as to show their maximally concentrated phase at mid-day rather than at mid-night (Brown and Webb, 1948, 1949), and hence the rhythms of activity of the two pigments are seen to be out of phase with one another as long as the reversed periods of illumination are continued.

It is also seen from Figure 2B, beginning at point *b*, that when the animals are returned to constant darkness after the three complete cycles of reversed illumination, the black pigment, as a result of a shift in the phase of the endogenous source of the rhythm, continues to exhibit a persistent 24-hour cycle of activity with maximum concentration of the pigment now at about 7 A.M. Under these conditions the red pigment also undergoes a similar rhythmical activity completely in phase now with the black. This rhythm was observed to persist unchanged for more than a week.

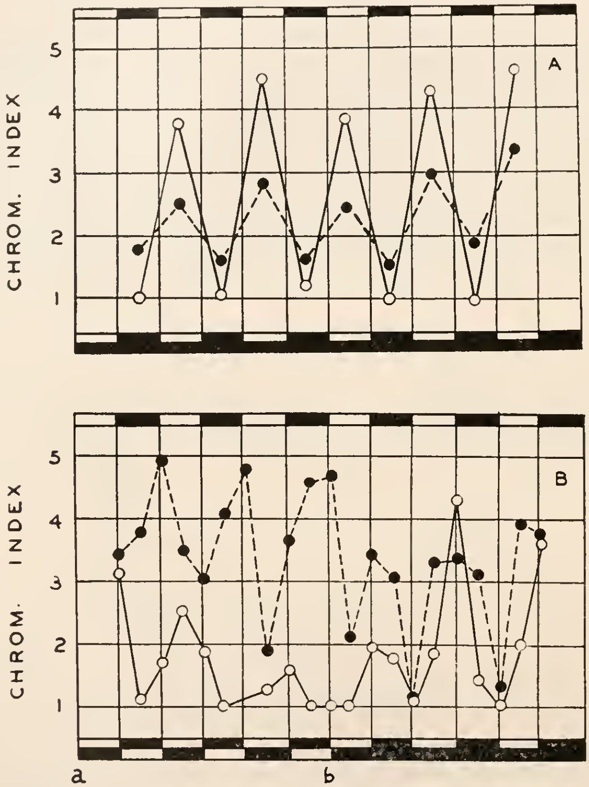

FIGURE 2. A. The normal persistent rhythmicity of red (solid line) and black (broken line) chromatophores in constant darkness. See text for explanation of diagrams.

B. Behavior of red (solid line) and black (broken line) chromatophores in response to a series of periods of reversed illumination beginning at *a*, followed by constant darkness beginning at *b*.

These results clearly indicate that the red pigment, other environmental stimuli removed, is under a very effective control by an endogenous mechanism of persistent rhythmicity, and that in the absence of such other stimuli the various phases of red chromatophore activity are controlled and experimentally shifted in a manner quite parallel to that found for the black chromatophores.

It is also evident from these observations that the red pigment is capable of exhibiting an activity independent of that of the black; indicating, in addition, some difference in the factors contributing to their control.

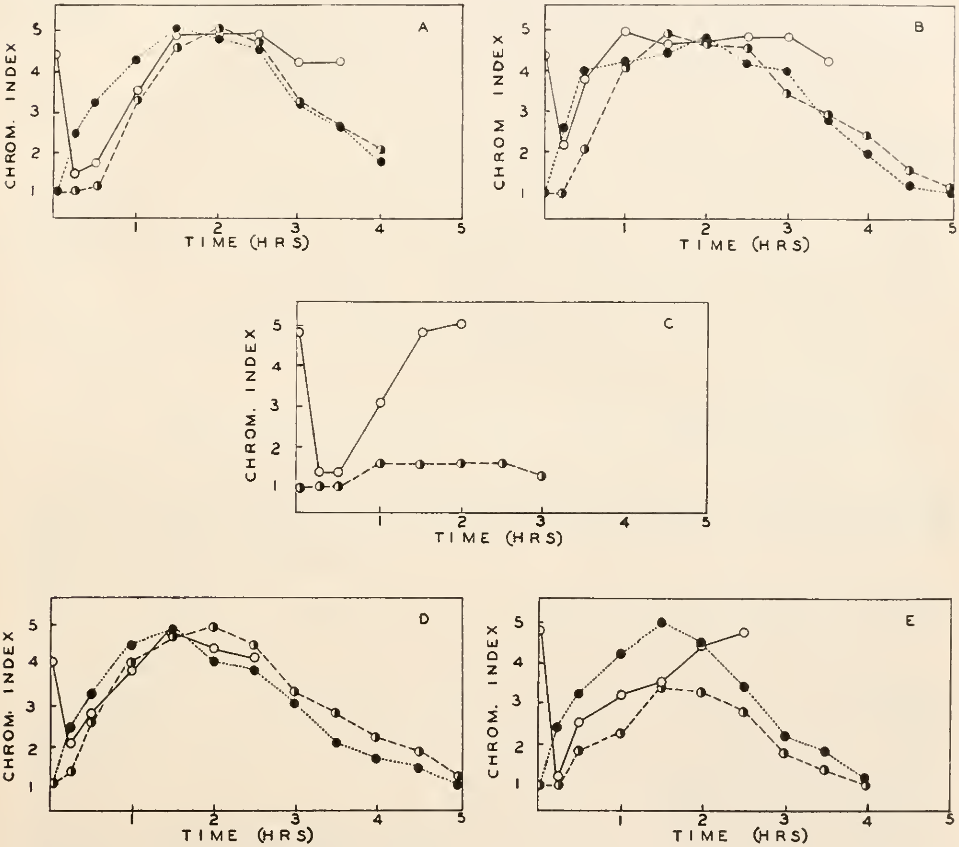

Figure 3. Responses of chromatophores of eyestalkless (red chromatophores—dashed line; black chromatophores—dotted line) and black-adapted intact *Uca* (red chromatophores—solid line) to injection of extracts of A. sinus glands, B. optic ganglia, C. circumoesophageal connectives, D. brain, and E. thoracic cord.

Sources of Red-Pigment-Influencing Factors

The sinus glands and various portions of the central nervous system appeared to be the most likely sources of chromatophorotropins influencing red pigment, in view of their demonstrated activities on white and black pigments in both this species (Brown, 1948) and another crab (Bowman, 1949). Consequently, a series of experiments was designed to determine what influences, if any, extracts of these organs might show.

Sinus glands, optic ganglia from which sinus glands had been carefully removed, brains, connectives, and thoracic cords were removed from a number of specimens and triturated in sea water in such volumes that a single dose (0.05 ml.) to be injected later into an assay animal would contain the equivalent of one-third of the complement of that tissue taken from a single animal. Such an extract was referred to as possessing a concentration of 1/3. The various extracts were then each assayed on (1) five *Uca pugilator* whose eyestalks had been removed 24 hours previously and whose red chromatophores were in an initial state of 1, and (2) five normal animals which were adapted to a black background and which were maintained upon a black background throughout the assay.

The states of the red chromatophores were determined at the time of the injection and then at 15 minutes, 30 minutes, and at 30-minute intervals thereafter until the chromatophores returned to their initial states.

The results of these experiments are summarized in Figure 3. The solid lines represent the averages for the black-adapted assay animals whose red pigment was initially dispersed, and the dashed lines represent the averages for the eyestalkless ones whose pigment was initially concentrated. The dotted lines show the behavior of the black chromatophores in the eyestalkless assay specimens.

It is readily seen that all of the extracts exhibited a substantial influence on the red chromatophores, an effect not shown either by sea-water injections or injections of extracts of muscle tissue which were used as controls.

The influence of all the active extracts showed two characteristics in common. The first was that they all exerted a rather strong concentrating influence upon the red pigment that was initially dispersed in the black-adapted assay animals. Undoubtedly the same type of activity accounted for the significant delay in the initiation of dispersion of red pigment in the eyestalkless assay animals whose red pigment was initially strongly concentrated. There is, in this latter connection, a rather close correlation between the magnitude of the red-pigment-concentrating action and the extent of this initial delay.

The second type of activity, one which was most conspicuously evident in the eyestalkless assay animals, was that of strong dispersion of red pigment. This action was also evident in the black-adapted animals in the form of the dispersion of the red pigment to a condition greater than that of the initial and final states, following the initial period of concentration.

Closer inspection of Figure 3 shows that the five extracts did not exhibit equal red-pigment-concentrating actions on the black-adapted assay animals. It can be seen that the apparent order of decreasing activity in this regard was as follows:

Thoracic cord > Connectives > Sinus glands > Brain > Optic ganglia

The foregoing order of red-concentrating activity was not the same, or even the simple inverse, of the order of red-pigment-dispersing activity which appears to be:

Optic ganglia > Brain > Sinus glands > Thoracic cord > Connectives

It would appear from these data that all of the portions of the nervous system, and the sinus glands as well, possess two different principles which are responsible for these two observed influences on red pigment.

Additional support for the dual activity of brain and thoracic cord extracts upon red pigment is seen in Figure 4, A and B, in which extracts of these organs were

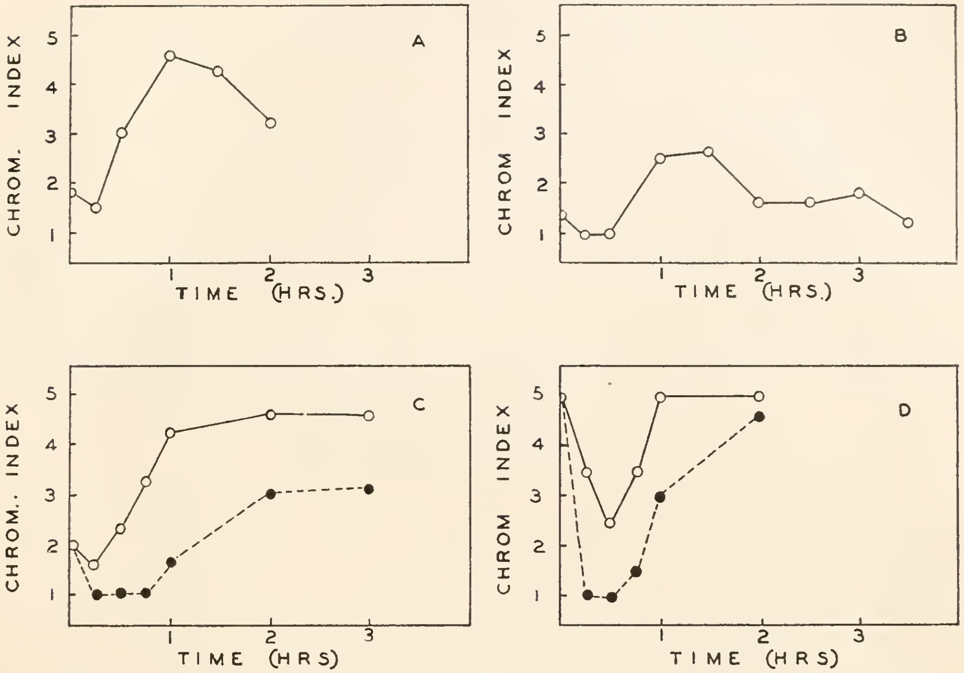

FIGURE 4. Responses of intact white-adapted *Uca* to extracts of A brain and B thoracic cord. C. Responses of the red chromatophores of eyestalkless *Uca* to alcohol-soluble (broken line) and alcohol-insoluble (solid line) fractions of sinus glands. D. Same as C but now responses of intact black-adapted *Uca*.

injected into normal white-adapted assay animals maintained upon a white background. Again, the same duality of activity of the extracts is observed.

A partial separation of these two principles appears to have been effected by the preparation of two types of extracts of sinus glands: one an ethyl-alcohol-soluble fraction and the other an ethyl-alcohol-insoluble fraction. Figure 4, C and D, shows the results of injecting these two fractions in equivalent concentrations into (C) eyestalkless assay animals selected to show slight red dispersal, and (D) black-adapted assay animals. In both instances the alcohol-soluble fraction appeared to possess a distinctly larger proportion of the red-pigment-concentrating activity, and the alcohol-insoluble fraction to possess the more black-dispersing action.

In an attempt to compare quantitatively the various portions of the nervous system and the sinus glands, one with another, with respect to their red-dispersing activity, extracts of sinus glands and of optic ganglia with sinus glands carefully removed were made up in concentrations of 1/3 as in the earlier experiments. A series of dilutions of these extracts was made to yield concentrations of 1/3, 1/9, 1/27, 1/81, 1/243, and 1/729. Each concentration of each organ was injected into five eyestalkless assay animals. This experiment was repeated three times. In Figures 5, A and B, are shown the relationships between the log concentration of the extract and the red-pigment-dispersing activity. The relationship is seen

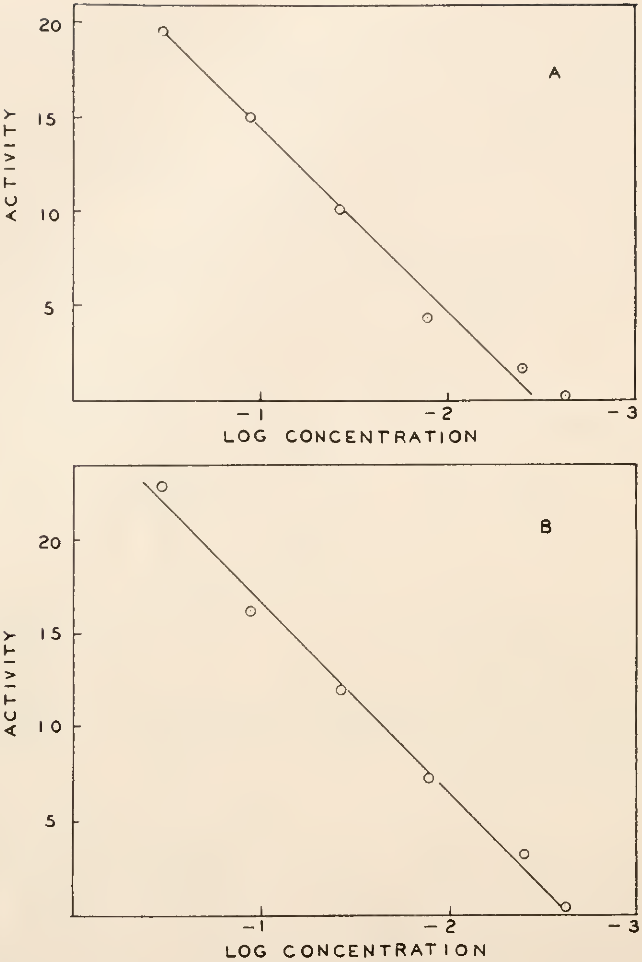

Figure 5. Relationship between extract activity on red chromatophores and log of concentration. A. Sinus gland extract. B. Extract of optic ganglia.

to be essentially a simple linear one. The activity was determined in the manner described by Brown and Sandeen (1948) in which the total of all the average chromatophore readings at 15 minutes, 30 minutes, and at 30-minute intervals thereafter until the end of the effect are added together. The difference between this sum and the corresponding sum at the same time intervals which would have indicated no activity was considered a reasonable measure of the activity since it incorporated both the amplitude and duration of effect of the injected extract. Using this dilution-series information to indicate the relationship between the concentration of the principle concerned and the amount of activity, and then using values of activities calculated in a similar manner from the data of Figure 3, it may be estimated that the optic ganglia and brain both contain approximately the same quantity of the principle. The sinus glands appear to possess only about one-half as much as the brain. The thoracic cord contains about one-thirteenth the amount of the brain, and the connectives only about one-eighty-eighth of the amount in the brain.

It must be borne in mind that these values are still quite rough approximations in view of the calculations being based on the assumption that the amounts of the antagonist (red-concentrating substance) are in equal quantities in all portion of the nervous system, an assumption that is in all probability not justified.

Similarly, values for the relative quantities of red-concentrating principle within the various organs tested assume equal quantities of its antagonist (red-dispersing factor) in all portions.

More precise determination of the quantitative distribution of the two factors within the various tissues must obviously await the development of methods for the complete separation of these from one another.

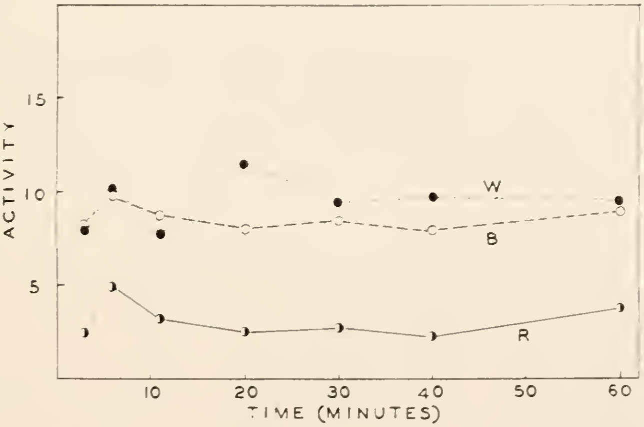

FIGURE 6. Alteration in activity of sinus-gland extracts on red (R), black (B), and white (W) chromatophores over the course of one hour following extraction.

ON THE PROBABLE IDENTITY OF THE BLACK- AND RED-DISPERSING PRINCIPLES

All the data which have been obtained in the course of these experiments upon the red chromatophores support an hypothesis that the red-dispersing principle from the sinus glands and the central nervous system is identical with the black-dispersing principle from the same organs. Two types of experiments have provided particular support.

In the first of these experiments, when eyestalkless assay animals were injected with extracts of various known sources of red-dispersing principle, the sinus glands, thoracic cords, brains, and optic ganglia, extracted in such proportions as to provide concentrations of 1/3, the effects upon the black chromatophores were observed simultaneously with those on the red. As can be seen from the results shown in Figure 3, A, B, D, and E, the action on the red chromatophores parallels in a remarkable fashion the action upon the black after the initial period of retardation. This initial retardation of dispersal has been seen earlier to be due to the simultaneous presence of a red-concentrating factor. Following the retardation the red pigment usually overtakes the black, for which there has apparently been no similar concentrating principle present, and then the duration of the action of the extracts upon these two pigmentary types is virtually identical.

The second type of experiment involved a determination of the alteration with time of the activity of extracts of the connectives of *Uca* upon three chromatophore types. Extracts of connectives were made up to provide concentrations of 1/3. Five assay animals were injected after 3, 6, 11, 20, 30, 40, and 60 minutes and the activity of the extract determined at each of these times on the red, black, and white pigments. Figure 6 shows the average of the results of three such experiments. The parallel character of the activity of the extracts upon the red and the black chromatophores is conspicuous. In contrast with this, the relationship with respect to the white pigment appears to be different from those of the red and black, and other types of evidence have clearly indicated them to be two distinct substances.

Summary

1. The red chromatophores of *Uca pugilator,* when other factors are equal, exhibit extensive responses to background. They disperse their pigment upon a black background, and concentrate it upon a white one.

2. Under conditions of constant darkness the red chromatophores show a striking persistent daily rhythmicity, dispersing their pigment by day, and concentrating it by night.

3. Extracts of sinus glands, and all of the major portions of the central nervous system, possess strong activity upon the red chromatophores. All portions exhibit two types of activity due to the possession of two principles. One of the activities is red-concentration and the other, red-dispersion.

4. The action of the red-concentrating principle can dominate the response when there is, concurrently present, high concentrations of both red-dispersing and red-concentrating principles.

5. The red-concentrating activity of all extracts is of much shorter duration than is the red-dispersing activity, the latter often lasting 3 to 4 times as long as the former.

6. Evidence is presented which strongly suggests that the black-dispersing and red-dispersing actions are due to one and the same principle.

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THE FATE OF INDIA INK INJECTED INTRACARDIALLY INTO THE OYSTER, OSTREA VIRGINICA GMELIN

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The contributions of Metchnikoff and others to the problem of the role of the phagocyte in the "defense" reactions of invertebrates as well as vertebrates is well summarized in the book, "L'immunité dans les maladies infectieuses" (1901). His concept of the relationship between intracellular digestion in the lower forms of life and phagocytosis in higher forms of organisms still merits the attention of those interested in the evolutionary aspects of the origin of "defense mechanisms." Indeed, the role of the amebocyte in the digestive functions of the oyster has been outlined by several workers (Vonk, Yonge, Takatsuki, Nelson, Chestnut) and its role in "defense" suggested by recent findings of Nelson (personal communication) and Stauber (1945). No careful delineation of the role of the amebocytes[2] of the oyster as a "defense" cell has yet come to the author's attention. Takatsuki's paper on the nature and functions of the amebocytes of *O. edulis* is of real value but stressed the digestive aspects though several pertinent findings of his will be discussed later. On the contrary, the phagocytosis of the gymnospores of the gregarine, *Nematopsis ostrearum* Prytherch, by the amebocytes of the oyster *O. virginica* and the subsequent development of the parasites within these cells is clear evidence of their failure in this case to act as defense cells. This does not constitute proof, however, that the amebocytes are not active in defense since examples might be chosen for citation of phagocytic cells being (1) host cells for parasites (Leishmania in man or hamster, Meleney, 1925; and Sphaerita in commensal protozoa, Wenrich, 1944), (2) disseminators of stages of parasites resistant to digestion (anthrax spores in frog lymphocytes, Trapeznikoff, 1891), (3) host cells for one stage in the life cycle and not for the other stage (cryptozoic *vs.* erythrocytic stages of avian malarias, Huff and Coulston, 1944), or (4) host cells in one anatomical or physiological situation and not in another (zoochlorellae in Tridacna, Yonge, 1944). The cases cited do not constitute proof that intracellular digestion is an impossible achievement in the phagocytic cells of the organisms mentioned.

The use of injected dyes greatly aided investigators in describing the phagocytic cells and their role in local and general inflammatory processes. The papers of Buxton and Torrey (1906) are an especially interesting series of studies on the disposition and fate of metabolizable as well as non-metabolizable particulate matter injected into mammals. Cuenot's report (1914) on the phagocytic organs of molluscs lacks any mention of the oyster. Furthermore, although he used parenteral injections of particulate matter like ink, he was concerned only with their immediate removal from the circulation, not their ultimate disposal by the mollusk. Takatsuki (1934) studied the fate of ink and carmine injected into the body of *O. edulis*. His findings, in general, are in agreement with those in the present report. Unfortunately, he fails to include in his report the details for area and amount injected, time and temperature relationships, etc.

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[1] The author is deeply indebted to Dr. T. C. Nelson for his many suggestions and generous support of this work.

[2] Amebocyte, leucocyte and phagocyte are used interchangeably. The cells under discussion are found free in the blood and are both amoeboid and phagocytic.

Ranson's (1936) findings were based on the exposure of whole living oysters to a sea water medium containing neutral red, or emulsions of aniline oil and other oils or coal tar. Since the absorptive surface in these cases was apparently the whole of the exposed epithelial layer of the oyster, even though he describes a leucocytosis and mass migrations of leucocytes across epithelial layers, his observations are clearly corollary to the general object of the work begun here.

The present study was undertaken as the first step in learning the nature, extent and action of the "defense" processes in the oyster. The results reported herein constitute a clear-cut demonstration of the role of the oyster amebocyte, or phagocyte, in the disposal of large masses of particles of india ink following intracardial injection. Subsequent steps should include studies using similar injections of non-pathogenic and, if possible, pathogenic (for the oyster) organisms as well as the studies of the responses of the oyster to the local parenteral injection of a similar series of particulate matters, non-living and living.

Materials and Methods

A group of oysters was filed down with a wood rasp on the area of the left valve over the pericardium. When the shell had been worn through by the rasping, the pieces of thin shell were picked away with forceps exposing the whole pericardium. This technique was previously found to leave an undamaged oyster (Stauber, 1940) capable of living and functioning for a long time in an aquarium. The filed oysters in this series were held in the aquaria a long enough period for the mantle to lay down a thin prenacreous layer over the area exposed.

On the day of injection the new prenacreous material was picked away. Since preliminary experiments had demonstrated that the heart could not be hit through the intact pericardial wall on every occasion, the pericardium was opened to expose the heart for injection. A dropping pipette with a finely drawn out tip was used for the injections. This was partly filled with a 1 : 10 dilution of Higgins india ink in sea water and inserted into the ventricle of the oyster. Partly by the warmth of the author's fingers and partly by pressure on the bulb, amounts of diluted ink from 0.25 to 0.45 ml. were injected. The rule was to inject as much as possible under the conditions of the experiment. The injection period usually was terminated when the oyster heart became so firmly contracted that further injection of material caused escape of the ink through the puncture wound.

For the type of study reported here and because of the difficulty of estimating the weight of the oyster meat in the intact animal, no attempt was made to quantitate the mass injected on the basis of the body weight of the oyster. Since india ink precipitates in sea water, loose aggregates of various sizes were injected, some macroscopically visible. Every attempt was made to reduce these in size by shaking before injection but often reagglomeration took place in the injection pipette during the period of injection. The question of size of particle injected may be of great importance in the immediate distribution of ink observed and probably further trials should be run using other materials for injection with more nearly uniform, smaller-sized particles for comparison (see Cuenot, 1914). Though the general picture reported here would be altered somewhat by the particle size introduced, it seems probable that the chief difference would have been an earlier, more widespread distribution of the ink.

After intracardial injection the oysters were returned to aquaria until the appropriate interval had passed (Table 1). They were then opened, the meat carefully shucked out and placed whole in fixative (Zenker-formol 5 per cent) for approximately 15 minutes, then removed to a glass plate and carefully divided into portions by cutting in the transverse plane at right angles to the long axis of the oyster. The pieces were then placed in suitable amounts of fresh fixative for 24 hours before washing. After mounting in paraffin, sections were cut at 7 μ and stained with hematoxylin and eosin.[3]

TABLE I

Record of amounts of ink injected intracardially into the oysters and the length of interval between injection and killing time

Oyster No.	Am't of india ink injected	Interval between injection and fixation
1	0.25 ml.	15 min.
2	0.3	1 hour
3	0.45	2 hours
4	0.25	4 hours
5	0.4	22 hours
6	0.25	8 days
7	0.25	17 days
8	0.25	25 days
9	0.45	33 days
10	0.25	42 days

The water temperatures in the aquaria in which the injected oysters were held varied more or less directly with outside temperatures during the period. Since the period covered by the investigation was from April 8 to May 20 water temperatures in the aquaria rose irregularly from 12° to 21° C.

In studying the tissues the several sections of each oyster were studied microscopically, notes being made concerning the distribution of the ink both intracellularly as well as extracellularly, under the medium power as well as under the oil immersion objective. When the whole series had thus been observed key locations were critically reviewed for each oyster. The types of information tabulated may be grouped in three categories: (a) the presence or absence of ink in blood vessels or sinuses of representative areas, (b) migrations of phagocytes containing particles of ink through representative epithelia and (c) presence or absence of ink in various lumina and cavities. The following blood channels were sampled: (1) the ventral pallial artery of the mantle, (2) a sinus of the same area of the mantle, (3) the anterior aorta seen in a typical section through the visceral

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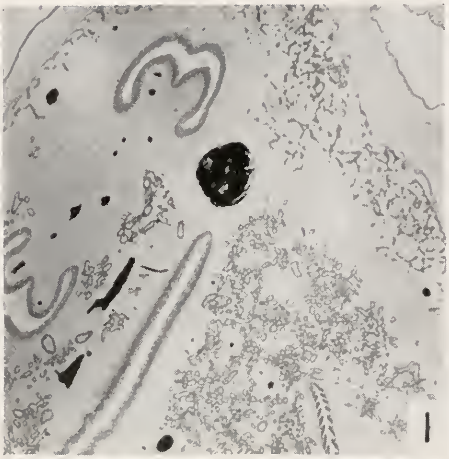
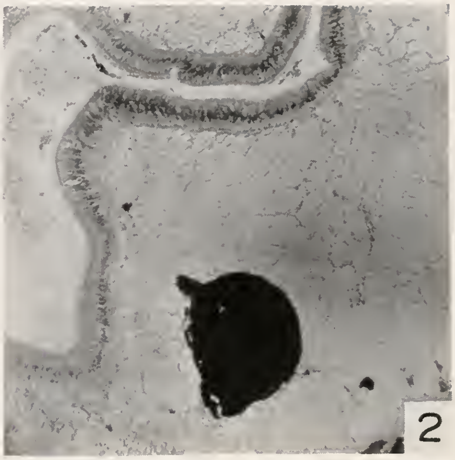
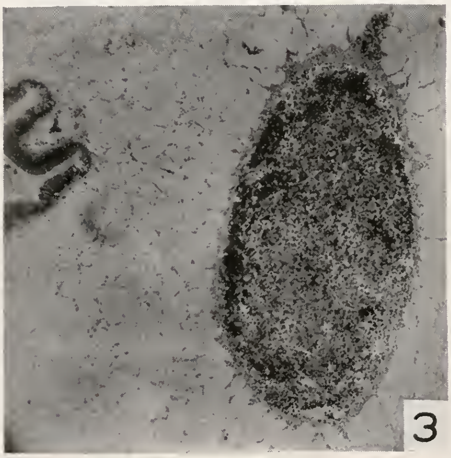
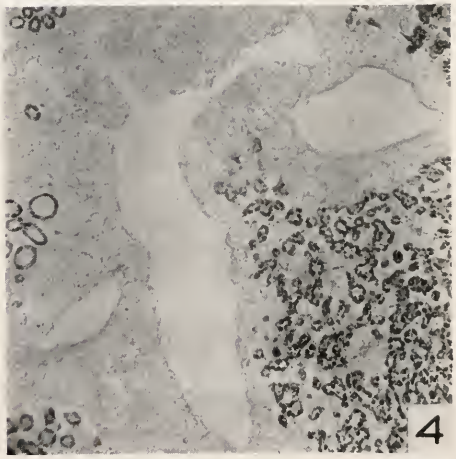
[3] The author is indebted to Miss Deborah Jackson and to Dr. James Shannon, of the Squibb Institute for Medical Research, for preparation of these tissues for microscopic examination.

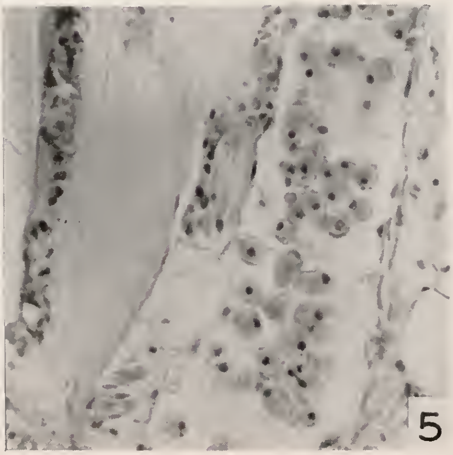
Plate I

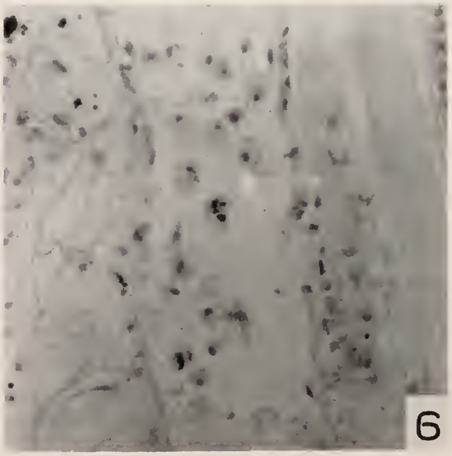












mass, (4) the largest sinus in the visceral mass cross section, (5) a small artery of the visceral mass, a subbranch of one of the hepatic arteries approximately 0.1 mm. in diameter, (6) a small sinus of the same area and magnitude for comparison, (7) an artery in the adductor muscle, (8) one of the posterior gastric arteries in the region of the so-called oral process ventral to the adductor muscle, (9) the heart, (10) the medial gill axis sinus, (11) a lateral gill axis sinus and (12) two vertical gill vessels, one leading from the medial aspect of the inner demibranch toward the medial gill axis sinus and the other from the lateral aspect of the same demibranch to the lateral gill axis sinus. In any area wherever enough phagocytes could be seen (50–100) percentage counts of those containing ink granules were recorded.

Migrations of ink-laden phagocytes through epithelial layers were sought by comparing equal numbers of representative fields from similar areas of the anatomy of the oyster. Except where noted, five consecutive fields were examined for migrating ink-laden phagocytes. The following sites were chosen as representative: (1) mantle epithelium facing the outer palp, (2) outer palp epithelium on side facing the mantle, (3) medial aspect of outer palp facing the adjacent surface of the inner palp on the same side, (4) lining of the promyal chamber near the point where it becomes contiguous with the suprabranchial chamber, (5) lining of the stomach in the area of its greatest diameter, (6) mid gut epithelium in three other locations, two areas in the visceral mass proper, and one in "oral process," (7) epithelium in the rectum dorsal to the adductor muscle, (8) a series of ten cross sections of tubules of the digestive diverticula, (9) gonaducts in region of visceral mass, (10) nephridial tubules, (11) external lining of the heart and (12) internal lining of the pericardium.

Ink, either free or in phagocytes, was sought in the lumina of five portions of the digestive tract mentioned above, in the lumina of the ten tubules of digestive diverticula examined, in the promyal chamber, in the portions of the infrabranchial

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PLATE I

FIGURES 1–6.

FIGURE 1. Oyster No. 3. 2 hrs. after injection. Section through anterior end of visceral mass near the mouth. Anterior aorta and many smaller arteries are occluded with ink. Note absence of ink in large and small sinuses. × 15.

FIGURE 2. Oyster No. 1. 15 min. after injection. Section through tip of visceral mass ventral to the heart. Large and small arteries near the style sac are shown occluded with ink. × 50.

FIGURE 3. Oyster No. 6. 8 days after injection. Section through anterior end of visceral mass. Large aorta occluded by leucocytes laden with ink. × 50.

FIGURE 4. Oyster No. 9. 33 days after injection of ink. Section through anterior end of visceral mass dorsal to palps. Anterior aorta and a large visceral sinus shown. Neither contains any significant numbers of leucocytes. × 50.

FIGURE 5. Oyster No. 4. 4 hours after injection. Section through outer left lamella of outer left demibranch showing a vertical gill vessel. No ink seen in amebocytes; the single dark body in each amebocyte is the nucleus. × 625.

FIGURE 6. Oyster No. 6. 8 days after injection. Section through the inner lamella of the left inner demibranch near the gill axis and flanking the left suprabranchial chamber. A vertical gill vessel is shown containing ink-laden amebocytes. Similar ink-laden cells are also seen extravascularly. × 550.

chamber between palp and mantle and between the palps, in the lumina of nephridial tubules and gonaducts and in the pericardial cavity.

Results

The response of the oyster to the intracardial injection of india ink, under the conditions listed above, may be conveniently divided into four phases: arterial occlusion by the agglomerated masses of ink, phagocytosis of the ink particles, distribution of the ink by the phagocytes and elimination of the ink from the oyster. It is important to note that there was a considerable overlapping in timing of the sequence of these events.

Arterial occlusion. The immediate effect of the intravascular injection of agglomerating particles of india ink is virtual embolism of the arterial system of the oyster. The arterial system as reported by Leenhardt (1926) and Elsey (1935) is clearly outlined in this way. Even while injecting the suspension of ink the anterior and posterior aortae are seen. The arterial branches and sub branches to visceral mass (hepatics and posterior gastrics and anterior ventral), palps, mantle, adductor muscle and rectum are very noticeably blocked in the gross as well as in section with aggregations of free ink particles (Figs. 1, 2 and 9). If the lacunae, or sinuses, in areas near the arteries are examined they are usually found relatively free from blood cells and except for an occasional free granule or two, no ink is to be seen (Fig. 1). The gill axis sinuses are likewise devoid of cells and ink. Other gill vessels may show more blood cells but little or no ink (Fig. 5). The arterial emboli are first composed of ink alone (Fig. 2). From the 22nd hour to 8 days after the injection of the ink the emboli are continued by masses of phagocytes most of which have engulfed particles of ink (Fig. 3). Later, between the 8th and 17th days, with the dispersal of the ink-laden phagocytes the arteries appear as free from cells (Fig. 4) as do those of untreated oysters.

Phagocytosis. No unquestioned phagocytosis of ink was noted at the 15 min. interval (oyster No. 1) though it would easily have been obscured in the ink-filled arteries. Little or no phagocytosis was noted anywhere before the fourth hour (oyster No. 4). Occasionally, as in the gill axis vessels of oyster No. 3 at 2 hours, where numerous blood cells were seen, 1–2 per cent contained a few granules of ink. Elsewhere in the same oyster phagocytes with ink were even rarer.

Beginning with oyster No. 4 more phagocytes were seen in the arteries and more contained ink, though the distribution may be variable. For example, there were many phagocytes containing ink in arteries in the adductor muscle or visceral mass but few with ink in the ventral pallial artery.

By the end of the first day after injection (oyster No. 5) mass accumulation of phagocytes in the arteries was observed. Furthermore, almost all the ink is intracellular in those phagocytes which continue the embolic obstruction of the vessels, with most of the phagocytes containing ink granules (see Fig. 3, oyster No. 6 for a similar condition). In direct contrast to this picture, although blood cells are seen in vertical and gill axis sinuses or sinuses of viscera and mantle, the percentage of phagocytes containing ink particles ranges from 2 per cent in a lateral gill axis and a small sinus of the visceral mass of this oyster (No. 5) to 15 per cent and 19 per cent in vertical gill sinuses.

Subsequently ink is only occasionally seen free; the granules are observed almost entirely in the cytoplasm of the phagocytes. The presence of a few ink granules in blood vessels throughout the later period (oysters No. 9 and No. 10) is possibly due to its release from phagocytes upon their death or through "defecation" by active phagocytes (Chestnut, thesis), although there is no direct evidence to support this.

The origin of the phagocytes which engulf the large amount of ink injected is an important point to be considered. It should first be noted that even in the untreated oyster "the origin and mode of production of amebocytes are not known; no definite organ is known to produce them" (Takatsuki, 1934). It is well known and easily observed that there is a large mobile reserve of phagocytic cells. Although few of these are seen within the arteries or even in the larger blood sinuses, many are found in the smaller sinuses of gill, mantle and viscera. Probably most, however, are scattered in the smallest lacunae or intercellularly especially under the epithelium of the digestive tract, digestive diverticula and gonaducts. Their role in digestion has already been referred to and numerous migrations of phagocytic cells in the epithelia can easily be noted in oysters (Fig. 9).

It is unlikely that mitosis of pre-existing phagocytes plays a predominate role in producing (or mobilizing) the phagocytes which dispose of the ink since, of the many phagocytes examined, only one has been observed in mitosis. This is very similar to the role of hematogenous lymphocytes and to a lesser extent monocytes as described by Maximow (1927) for the local inflammatory process in the subcutaneous tissues of the rat injected with trypan blue (Taliaferro, 1949). Since careful study of the arterial walls does not disclose mass migrations of phagocytic cells through them from the surrounding tissues their arrival at the ink masses via the hematologic route is presumed. This is understandable since, although obstruction of the artery in Figure 2 is complete, this is in an oyster *after* fixation and shrinkage. The shrinkage observed is believed not to be merely a function of the fixative used as found by Orton (1937) in his study of the effects of Bouin's fixative on isolated 0.1 gm. pieces of oyster tissues, but also of the blood loss of the cut oyster. Thus, it seems probable that blood flow did not altogether cease and that the slight trickle of fluid through these almost occluded channels made possible the appearance of the phagocytes in the ink-filled areas of the vessels. An alternative, acceptable explanation might be the migration of the phagocytes to the ink-filled arteries by their own activity. This would be possible even in a direction counter to the current (incomplete occlusion) if the current were weak,[4] and if the phagocytes moved along the blood spaces using the lining as a substrate for amoeboid activity. The usual picture of finding blood cells almost exclusively in such a position in the larger vessels may be offered as evidence, though Cuenot believed cells similarly placed on the walls of the small arteries of the "liver" of the amphineuran, *Acanthocites discrepans* to be fixed phagocytes. In the case of the oyster their relative inconstancy of number and position mark them as free phagocytes. The net result by either means would be the arrival in the blocked areas of large numbers of phagocytic cells.

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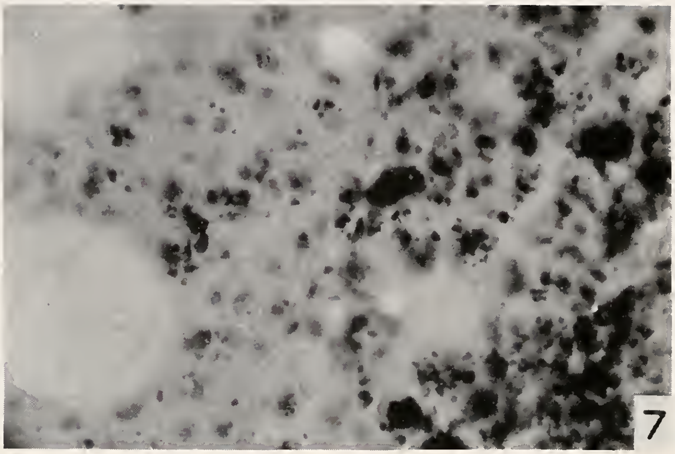
[4] The studies of T. C. Nelson (personal communication) and Hopkins (1934) show much reversal of current, much surging back and forth of blood in the vessels, especially in the mantle.

Plate II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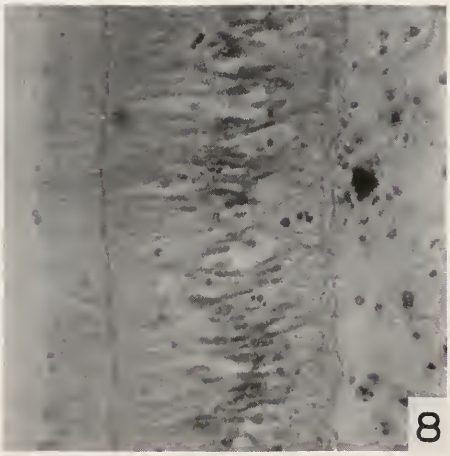
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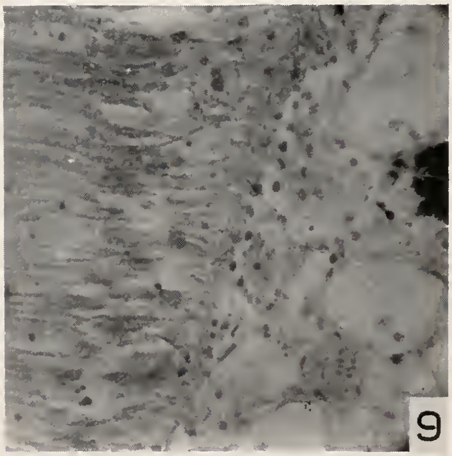
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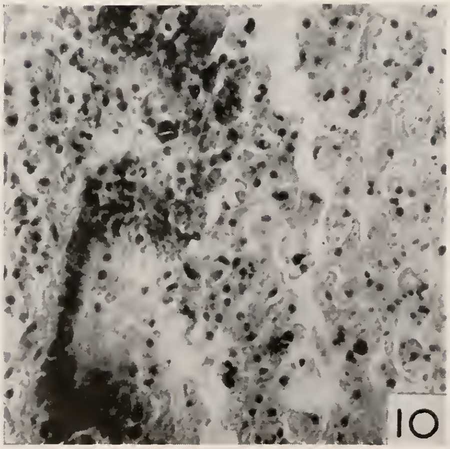
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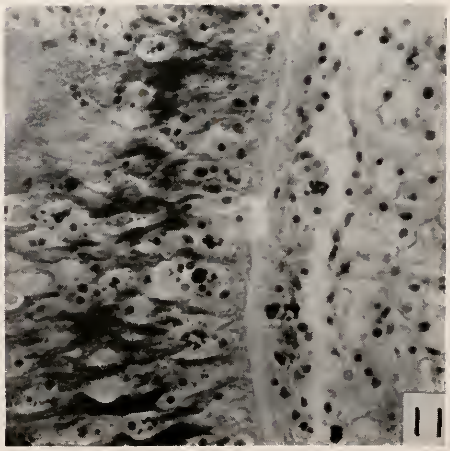
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Takatsuki discusses the types of blood cells seen in *O. edulis* and recognizes two main types: granular leucocytes and hyaline leucocytes. No data are available on the proportionate numbers of these two types of cells in oysters or on the relative phagocytic properties of the two types of cells. Indeed, confirmation of his findings is desirable since the hyaline leucocytes are claimed to be non-nucleate and not observable in stained preparations. Since the granules in the granular leucocytes are not stained by hematoxylin-eosin the relative importance of the two cells in phagocytosis is not known, except that the only figures in Takatsuki's report which certainly identify the cell type and also show ingested particles are granular leucocytes.

Distribution. Ink-laden phagocytes become distributed throughout the oyster following engulfment in the arteries. This is accomplished in two ways: first, by the passage of the cells from small arteries to sinuses in the usual fashion, and second, by direct migration through the walls of the blocked arteries both large and small. Both processes proceed actively. For example, the large increase in the proportion of ink-laden phagocytes in the gill axis sinuses (from 2–19 per cent in oyster No. 5 to 35–50 per cent in oyster No. 6) is indicative of the first process and Figure 7 clearly shows the migration of ink-laden phagocytes through the wall of a hepatic artery in the viscera of oyster No. 6. This phenomenon was first seen in oyster No. 5 (22 hours). It was characteristic of the findings for oysters No. 6, No. 7 and No. 8 even though occlusion of the arteries was no longer to be observed in oysters No. 7 and No. 8. Some migrations were still to be observed in oyster No. 9 but the process of migration through arterial walls was virtually completed between the 33rd and 42nd days.

The net result of these processes is the widespread distribution of ink-laden phagocytes so that wherever phagocytes are found in oysters after 8 days in this series, ink-laden specimens can be observed. This is in marked contrast to the distribution of ink in a mammal, like the rat, after intracardial injection. In the mammal the ink largely remains in the organs with macrophages strategically placed to remove material from the blood (spleen, liver, bone marrow).

PLATE II

FIGURES 7–11

FIGURE 7. Oyster No. 6. 8 days after injection. Section through wall of branch artery in visceral mass. Large numbers of ink-laden phagocytes seen in the lumen of the artery as well as many seen migrating through the wall of the artery. × 760.

FIGURE 8. Oyster No. 9. 33 days after injection. Section through wall of style sac showing migrations of ink-laden phagocytes. × 550 approx.

FIGURE 9. Oyster No. 1. 15 min. after injection. Section through style sac in approximately the same region as in Figure 8. Note the presence of a few amebocytes migrating through the epithelium of the style sac. Note also the artery occluded with ink. × 550.

FIGURE 10. Oyster No. 7. 17 days after injection. Section through palp region. To left is medial aspect of outer palp; to right is that portion of the infrabranchial chamber between the faces of inner and outer palps on the right side. Note the many ink-laden amebocytes on either side of the epithelium but especially in the infrabranchial chamber. × 600.

FIGURE 11. Oyster No. 9. 33 days after injection. Section through epithelium of intestine in the region of the oral process. Note the numerous ink-laden phagocytes migrating toward the lumen of this portion of the alimentary tract. × 550.

Takatsuki observed a similar widespread distribution of carmine-laden phagocytes in oysters injected into the body with that substance.

Elimination. The normal physiological movement of amebocytes through various epithelia is well known especially with reference to digestion (Yonge, Nelson, Takatsuki). It is not surprising then to find large scale migration of ink-laden phagocytes across epithelia into various lumina. Table II summarizes these findings. Note first that no migration of phagocytes containing ink was found until more than 22 hours had elapsed after injection of the ink. The first migrating forms with ink in them were seen in oyster No. 6 killed 8 days after injection and subsequent to the first migrations through the arteries. The epithelia of digestive organs (stomach, intestine, rectum, digestive diverticula) were the chief sites of the migrations (Figs. 8 and 11) though the presence of many ink-laden phagocytes between the palps on one side of oyster No. 7 (Fig. 10) or in the promyal chamber of oyster No. 10 indicates that the process occurred in a wide variety of epithelial sites. Migrating phagocytes were observed in the lining of both the pericardial wall and the outer aspect of the heart as well as in the pericardial space between these sites.

TABLE II

Migrations of ink-laden phagocytes through epithelia after intracardial injection of india ink

Oyster No.	Time after injection of ink	Number of ink-laden phagocytes observed in the epithelium†								
		Mantle, facing palp*	Palp, facing mantle*	Palp, facing palp*	Lining of promyal chamber*	Stomach*	Intestine visceral area*	Pericardial wall	Rectum*	Digestive diverticula**
1	15 min.	0	0	0	0	0	0	0	—	0
2	1 hour	0	0	0	0	0	0	0	—	0
3	2 hours	0	0	0	0	0	0	1	0	0
4	4 hours	0	0	0	0	0	0	0	0	0
5	22 hours	0	0	0	0	0	0	—	0	0
6	8 days	2	0	6	1	0	4	15	—	0
7	17 days	1	1	5	0	8	6	41	0	14
8	25 days	1	0	1	2	0	0	—	1	1
9	33 days	0	1	7	1	36	33	1	15	10
10	42 days	0	0	2	0***	20	8	6	2	9

— Area not observed.
0 No migrations observed.
* 5 oil immersion fields studied.
** 10 cross sections of tubules of digestive diverticula examined.
*** Many ink-laden phagocytes seen in the promyal chamber.
† The numbers are relative and of chief significance when compared with others in the same vertical column.

There were three sites in this series which, almost without exception, showed no migrating ink-laden phagocytes. These were the epithelia of the external (shell-secreting) face of the mantle, the gonaducts and the excretory tubules. Migrations through the epithelium of the gills were relatively rare though it is possible that this is a reflection of the initial distribution of the ink. While the primary occlusion of the arteries in the visceral mass, and the later migrations of ink-laden cells through the arterial walls of the visceral arteries, would account in part for the sites of migration observed, the failure to observe such migrations through gonaducts, excretory tubules and the external face of the mantle must be explained in some other fashion. Similarly, the presence of 35–50 per cent ink-laden phagocytes in the gill sinuses of oyster No. 6 is contrasted to the rare migrations of these cells across the gill epithelium. Takatsuki reported similar migrations of india ink or carmine-laden phagocytes across epithelia in various sites after injection of carmine into the body of the oyster, but his findings included migrations into excretory tubules and gonaducts. The discrepancy is not easily explained with the data available. Even his possible faulty interpretation through the use of the term "excretion" to include the voiding of undigested material from the body cannot explain the migrating leucocytes he observed. Nowhere in his report does he record the site of the injection of the carmine or ink. If the site had been into or in the vicinity of the excretory organ, a plausible explanation would be available; although, in this series, microscopic fields have been observed showing many ink-laden phagocytic cells just beneath the basement membrane of the epithelium of the nephridial organ without a single phagocyte in migration through the epithelium. Perhaps the frequency of migrations unrecorded by him through these sites is a partial explanation. In the series reported here, only a single ink-laden cell was observed traversing the epithelium of an excretory tubule. The absence of phagocytes from the lumen of the excretory tubules might be explained by the open pericardium since the wall of the auricle is claimed to have excretory functions (Ranson, 1936). This is especially true since we did observe ink-laden phagocytes in the pericardial cavity. In the case of the gonaduct, Takatsuki himself was at loss to explain such migrations. It is possible that his study was made in the autumn when the oyster amebocytes play an important physiological role in the involution of the gonad. Our study covered a period in the spring when the gonad was showing renewed activity. No migrations into gonaducts were observed.

The net result of these migrations was the voiding of ink into the lumina of organs leading to the outside. In fact, once the ink particles had passed through these epithelia they may be considered essentially as being "outside" of the body of the host. It is possible, of course, that some of these phagocytes (especially in the intestine and digestive diverticula and even on palp and gill surface) may migrate back into the oyster again if the ink present in their cytoplasms does not interfere with the performance of other functions. A net loss of ink would be expected, however, which would eventually lead to complete elimination.

Further evidence of this elimination was seen in the appearance in the aquaria of dejecta and rejecta of much darker appearance than that of uninjected oysters under similar conditions. Since no microscopic observations were made on these voided masses it cannot be stated whether the granules of ink were free or still in phagocytic cells when voided from the oyster. Chestnut (personal communication), after local parenteral injections of small amounts of india ink, states definitely that the ink in the feces was in phagocytes.

It will be noted from Table II that ink was still being eliminated from the oyster when the experiment was terminated, though the peak of elimination occurred on or about the 33rd day after the injection of ink. Oyster No. 10 was noted as having less ink present than any other oyster in the series. Although there are no supporting data available it is believed that the process would continue for a relatively long period of time with the curve of elimination gradually flattening out and with some ink still present, possibly for a year or more.

DISCUSSION

The process of elimination of india ink after intracardial injection is not confined to the oyster. Although no references to exactly similar work in mollusca have been found, vestiges of a similar mechanism for the removal of foreign bodies are described for mammals. Two related statements from Maximow and Bloom, "Textbook of Histology" (1939), are pertinent. Discussing free macrophages of the blood the authors state, "Many investigators have described macrophages in the blood. In animals injected intravenously with vital dyes or corpuscular matter, such as blood cells, large quantities of these substances accumulate in the free macrophages. They originate in the spleen, liver and bone marrow from fixed macrophages through contraction and isolation. They are found especially in the blood of the veins and of the right heart and the major part of them is filtered off in the capillaries of the lungs, but some may occasionally enter the general circulation" (p. 43). "If the macrophages have taken up indigestible particulate matter, as colloidal silver or india ink, they often degenerate; then the foreign particles are set free and may be taken up again by other macrophages. In the intestines and in the lungs the foreign particles may be eliminated from the body with the cells containing them" (p. 91). A local response of similar nature is described for the lungs. In this case, part of the carbon inhaled in city air is phagocytized and expelled in the so-called "dust cell," a macrophage of the lung. In the oyster, however, the process is more closely related to the intracellular phases of the digestive process.

TABLE III

Time sequence of events leading to disposal of india ink after intracardial injection into the oyster

Oyster number	Time after injection	Arterial occlusion	Presence of free india ink in blood vessels	Migrations of ink-laden phagocytes through arterial walls	Migrations of ink-laden phagocytes through epithelia
1	15 min.	+	++++	–	–
2	1 hour	+	++++	–	–
3	2 hours	+	++++	–	–
4	4 hours	+	+++	–	–
5	22 hours	+	+	+	–
6	8 days	+	±	++	+
7	17 days	–	±	++	++
8	25 days	–	±	++	++
9	33 days	–	±	+	++++
10	42 days	–	±	–	++

The numbers of pluses are approximate and relative and are significant only when compared with others in the same vertical column.

In a poikilothermic animal, like the oyster, the rates of various metabolic processes are greatly influenced by temperature. That the degree of activity of the phagocytes would be similarly affected by temperature is a logical conclusion. Therefore, the times reported here for phagocytosis, distribution and elimination of india ink (see Table III) must be considered in terms of the temperatures prevailing during the experiment. If the experiment had been conducted at 25° C. the events noted most probably would have occurred much sooner than recorded above.

A half century ago the responses of many organisms to the injection of foreign bodies, living and dead, were studied but the oyster was not included. It is strange that the oyster, an organism of such great value as a food in many parts of the world, especially in this country, and claimed to be scientifically the best known marine animal, has not been fully studied in this respect. Only a fraction of the oyster eggs fertilized each year grow to adult oysters. Many causes of mortality are known, predators like the starfish and oyster drill being especially important. There are always found, however, losses of unknown causation (Orton, 1924; and Loosanoff and Engle, 1941). Except for Tennent's work on *Bucephalus*, Prytherch's work on *Nematopsis* and Korringa's recent report on a fungal shell disease, few diseases peculiar to the oyster are known where the parasitic organism (not predator) produces direct effects on the living substance of the oyster (Dollfus, 1921; Orton, 1937). This is not meant to imply that *Bucephalus* or *Nematopsis* is necessarily a primary cause of mortality of the oyster. It is very unlikely that these are the only ones that do exist. The beginning study reported here is believed fundamental to a full knowledge of host responses upon which control measures might be based.

A similar series of oysters should be studied using foreign body particles of much smaller size. While arterial occlusion would probably be lacking and initial distribution of the ink less restricted, the phenomena of phagocytosis, migration and elimination would most likely be similar.

In the life cycle of *Nematopsis ostrearum,* a gregarine parasite the sporozoite of which normally develops in the phagocytes of the oyster, Prytherch notes that "though some of the sporozoites are evidently destroyed by the phagocytes a small number generally survive and grow rapidly at their expense." The mortality of sporozoites in a light natural infection was estimated at better than 50 per cent. Development of the sporozoite and spore formation in the oyster require about two weeks in warm weather, according to Prytherch. It is entirely possible that, in addition to the mortality mentioned above, there is an important sporozoite loss due, not to intracellular digestion by the phagocytes, but to migration and elimination of the sporozoite-laden phagocytes as in the case of the india ink granules described above. Indeed, unless the hypertrophy of the sporozoite-infected phagocyte restricts amoeboid activity, it is possible that the elimination of developed spores in this fashion may constitute the more normal route of infection for the crab host.

SUMMARY

The responses of the oyster to an intracardial injection of a sea water suspension of india ink were followed grossly and microscopically. The ink suspensions agglomerated readily and produced emboli which virtually occluded the arterial vessels of viscera, mantle and adductor muscle. Subsequent events, with considerable over-

lapping, were in sequence: (a) phagocytosis of the injected ink particles by mobile phagocytes, (b) distribution of the ink in the phagocytic amebocytes to all parts of the organism with concomitant resolution of the emboli and (c) eventual elimination of the ink from the organism by the migration of ink-laden phagocytes through the epithelial layers of the alimentary tract, digestive diverticula, palps, mantle, heart and pericardium into lumina from which they were voided. The epithelia of gonaducts, nephridia and shell-forming mantle were not routes of migration. A close relationship is noted between the role of the phagocytes in the normal digestive process and in the "defense" reaction to such a foreign body as india ink. The possible significance of the responses noted with respect to unexplained mortalities of the oyster is considered.

The author is deeply indebted to Dr. T. C. Nelson for taking Figures 2–4 and 7–11 inclusive.

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MULTICONJUGATION IN BLEPHARISMA

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Conjugation of three or more individuals, i.e. "multiconjugation," has been observed in mass cultures of *Blepharisma undulans.* The phenomenon in this species was first reported by Giese (1938a), and has been seen in a variety of other ciliates during the course of the last century (Chen, 1946). Chen, working on *Paramecium bursaria,* published the first detailed cytological study of multiconjugation. In the present paper an analogous cytological study is described for *B. undulans,* and experiments are set forth indicating how multiconjugation may be brought about.

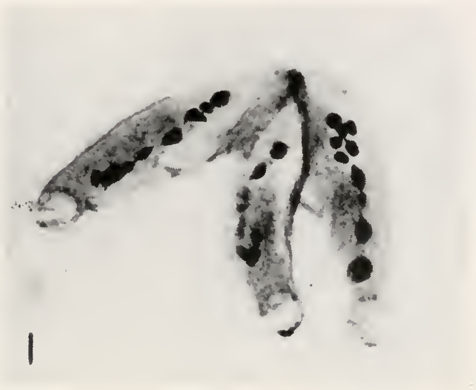
Two stocks of *B. undulans* have been employed, one from the Laboratory at Woods Hole, the other from Elon College, North Carolina. Both of these are morphologically equivalent to Giese's California stock (1938a; also below). The organisms have been cultured at room temperature, with filtered rain water as medium. A rich supply of bacterial food could be maintained in mass cultures by adding a barley seed to each culture dish. For cytological work, given animals have been put on slides, fixed in Carnoy, dried, and stained with methyl green without counterstain (Weisz, 1950).

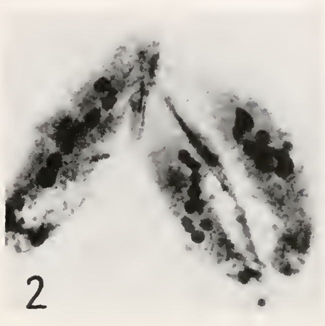
MULTICONJUGATION

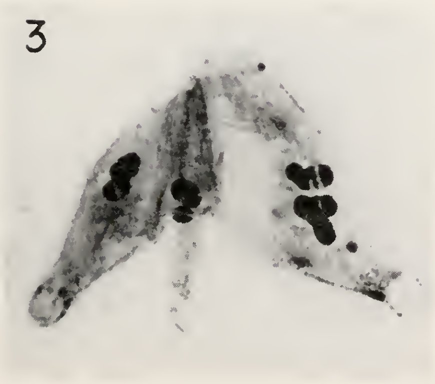
In dense cultures, conjugation epidemics have been observed at intervals, occurring almost simultaneously in different culture dishes and involving perhaps 90 per cent or more of all animals present. Rough counts have shown that approximately 5 per cent to 10 per cent of all matings seen were conjugations of three individuals. Occasionally four, and rarely five, individuals have been found joined into multiconjugant systems. All other conjugations have been paired matings.

Examination of a large number of "triples" has revealed two principal patterns of attachment among conjugants. In one pattern, the three participants are fused together firmly along the full extent of their peristome areas, i.e., from near the anterior tip of the animals almost to mid-level. Attachment is equally extensive between any two of the three members, and the trio suggests a tripod opened up in three dimensions. In another pattern, two of the three conjugants (A and B) are firmly and fully fused as above and as in paired mating, while the third conjugant (C) is peristomially attached to a small area of the peristome of either A or B. In these cases any direct physical connection is not evident between conjugant C and one of the other partners (cf. photographs by Giese, 1938a). Attachment of conjugant C to the posterior body regions of either A or B, as observed by Chen (1946) for *P. bursaria,* has not been seen in *B. undulans.*

In cases of matings involving more than three animals, a large variety of attachment patterns has been noted. Conjugant A might be joined to B, C to A, D to C and/or A, E (if present) to B, or to C, D, or A, etc. Attachment varies in extent but it is always peristomial. It would, therefore, appear generally that conjugative fusion in *B. undulans* always takes place along the peristome area, and that effective fusion need involve only a very small section of this area.







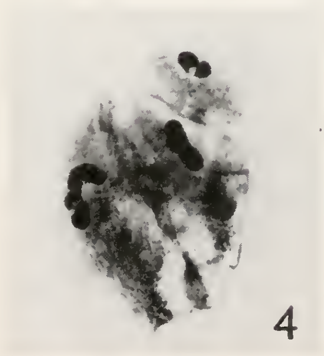


Plate I

Figures 1–4

Figure 1. Conjugation of 3 animals, prepared slide, Carnoy-Methyl Green. Animals are joined tripod-fashion. Soon after initial fusion; macronuclear chains beginning to contract. Appr. times 200.

Figure 2. Conjugation of 3 animals, prepared slide, Carnoy-Methyl Green. Left individual joined less extensively. Macronuclear contraction under way. Appr. times 200.

Figure 3. Conjugation of 3 animals, prepared slide, Carnoy-Methyl Green. Animals joined tripod fashion. After synkaryon formation; in each member two macronuclear primordia visible. Note "kernspalt"-like appearance of primordia. The smaller primordium in each member will degenerate. Appr. times 200.

Figure 4. Conjugation of 3 animals, prepared slide, Carnoy-Methyl Green. Animals joined tripod fashion. Late stage of conjugation. In each member only one presumptive macronucleus left; the latter in process of elongation. Appr. times 200.

Multiconjugation typically occurs among animals of equal size, although in many cases one (or more) of the conjugants is smaller than the others. Conjugative fusion lasts as long as in paired mating, i.e., approximately 3 days. After this time the participants separate in groups or singly within a few hours. All the exconjugants are viable and produce vigorous clones.

Cytological

Young (1939) has described in detail the essential cytological steps in normal conjugation of the Yale race of *B. undulans*. The Woods Hole and Carolina stocks differ somewhat from the Yale strain, particularly in the structure and the vegetative behavior of the macronucleus (Weisz, 1949), but the sequence of processes during conjugation is virtually identical to that in the Yale race (Weisz, 1950). The old macronucleus first condenses, fragments, and then degenerates, disappearing completely approximately 36 hours after the conjugants become fused initially. A synkaryon has formed in the meantime, and numerous readily visible, relatively large micronuclei now begin to appear. Simultaneously a number (typically four) of faintly staining macronuclear primordia arise. One of these enlarges, takes up an increasingly dense stain, passes through a "kernspalt"-like stage (Weisz, 1950), and ultimately becomes the definitive macronucleus of a new caryonide. The other primordia degenerate and disappear. During this phase the micronuclei become smaller, and throughout the remainder of the caryonide life history micronuclei are extremely small and often cannot be found at all. It is largely due to this circumstance that synkarya, and pregamic and postzygotic maturation stages cannot be reliably identified. The derivation of micronuclei and of macronuclear primordia from the synkaryon can, however, be inferred by experiment; experimentation has similarly brought out the temporal correlation between micronuclear and the visible macronuclear events (Weisz, 1950). Thus, for any given stage of the macronucleus, the micronuclear condition at that stage can be estimated. It should be noted parenthetically that in typical *B. undulans* only one macronuclear primordium persists, so that a caryonide is defined by the entire clone arising from an exconjugant.

In multiconjugation involving any number of conjugants, characteristic nuclear changes have been consistently observed in every one of the participating animals. This has been found true regardless of the specific pattern of peristomial attachment. The changes are always precisely identical in nature and duration to the cytological sequence observed in paired mating, and they occur synchronously in all members of a multiconjugant system. This is illustrated in Plate I, showing successive stages in the conjugation of three individuals. Since the old macronuclei disappear and new primordia develop, as in paired mating, it may be inferred that synkarya are probably formed in each of the members of a multiconjugant system. In this respect *B. undulans* and *P. bursaria* (Chen, 1946) are similar.

Experimental

In order to determine whether or not multiconjugation is attributable to the existence of multiple mating types, twenty-five single-clone cultures (including both Woods Hole and Carolina stock animals) have been prepared, and individuals from each clone have been mixed with individuals from every other clone. Animals from one of the clones to be tested have first been exposed to light for 1 to 3 days, a procedure which brings out depigmentation of the animals; organisms from another clone were left in the dark, ensuring the retention of the pigmented state (Giese, 1938b). Some 100 animals from each of the two clones have then been transferred together to a deep watch glass containing fresh, relatively food-free culture medium. Under such conditions the members of the two clones can be identified; moreover, transfer to new medium effects a fairly sudden reduction of food organisms, a condition necessary to induce conjugation (Giese, 1938a).

The mixing of clones in Blepharisma, unlike similar procedures in Paramecium, does not result in a striking mating reaction. Nevertheless, after some 20 to 50 hours more than 90 per cent of all animals present are involved in the mating process. This has been found true for mixtures of any two of the twenty-five clones studied. The result confirms similar observations of Giese (1938a). Inspection of each of the mixtures has shown that the majority of conjugation pairs consisted of members from only one of the clones, i.e., in perhaps 60 per cent to 70 per cent of the pairs both conjugants were either deeply pigmented or only lightly pigmented (some restitution of pigment having taken place in the interval). In the remaining mating pairs conjugants of different clones have been involved. In each of the denser mixtures, moreover, a number of multiconjugant systems has been found. Most of these consisted of three animals, usually derived from only one clone. Occasionally, however, four-animal and five-animal conjugations have been seen, and in some of these one or two of the participants derived from the second clone.

In a variation of these tests, organisms from a single clone have been transferred alone to fresh, food-free culture medium. As above, 1 to 2 days after transfer the great majority of animals present have been found in conjugation, and in the denser test cultures a number of multiconjugations could be observed.

Thus it is likely that both the Woods Hole and the Carolina stocks of *B. undulans* are selfing strains; that physiological differentiation into distinct mating types is absent, and that the occurrence of multiconjugation (as of paired conjugation) is primarily a function of food supply and of population density. The age of a clone does not appear to play a decisive role, since conjugation has been induced as early as in the 6th- to 10th-generation descendants of exconjugants.

DISCUSSION

A question of primary interest concerns the basic significance of the observed nuclear changes in multiconjugation. In one of the characteristic patterns in which conjugant A is extensively joined to conjugant B, and C is attached less extensively to B, does conjugant B exchange pronuclei with both A and C? From the manner of attachment it might appear probable that A and B exchange pronuclei as in paired mating, while an autogamous process takes place in conjugant C.

Unfortunately, evidence for the occurrence of autogamy in Blepharisma cannot, at present, be adduced by genetic means (as Sonneborn, 1939, has done for *P. aurelia*), in the absence of mating types, or of other demonstrably gene-controlled characteristics. Moreover, clear-cut cytological evidence for (or against) autogamy is, to date, not available for *B. undulans*, and this is not surprising in view of the inadequacy of the micronuclei for cytological investigation. Macronuclear events are always identical in all conjugative processes, and thus cannot be relied upon to differentiate between autogamy (if it occurs) and true cross-fertilization. Conceivably, an autogamous process need not necessarily be postulated for the second major pattern of multiconjugation, in which A, B, and C are joined in tripod-fashion, the extent of attachment being mutually equivalent among the members. In these cases a pattern of pronuclear exchange might occur, involving all three participants; but the possibility of autogamy is by no means ruled out. In multiconjugations involving four and five individuals, attachment of some of the members is often exceedingly insecure and superficial; these members separate off upon slight agitation very soon after they are initially joined, yet normal conjugative macronuclear changes have been observed in a number of such separated individuals. For these, at least, there probably can be no question of nuclear exchange.

Thus, although direct proof is lacking, the very occurrence of multiconjugation in *B. undulans* probably provides presumptive evidence for the occurrence of autogamy. If so, multiconjugation in this species is cytologically wholly equivalent to this type of "mating" in *P. bursaria* (Chen, 1946).

SUMMARY

A cytological and experimental study was carried out on conjugations involving more than two individuals of *Blepharisma undulans*. Attachment of the participants in a conjugating group occurs in a number of characteristic patterns. Regardless of the attachment pattern, nuclear changes occur in all members of a multiconjugant system. These changes are identical in nature and duration to those observed in normal paired conjugation, and occur synchronously in all participants of the system. The stocks of *B. undulans* used are found to be selfing, physiologically not differentiated into mating types. Multiconjugation can be induced by varying food supply and population density. The probability of autogamous processes in connection with multiconjugation is discussed.

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THE EFFECT OF X-RADIATIONS ON SQUID LARVAL TROPISMS

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The eye of the squid, *Loligo pealli*, is structurally very much like that of the higher vertebrates except that the sensory cells of the retina are directly exposed to the light source (Fig. 1). The eyes are among the first organs to differentiate, and the squid embryos manifest positive phototropism and negative geotropism long before the time of hatching. The chromatophores of the squid embryo are relatively very large, and appear and function at a very early stage. It seemed of value, therefore, to determine the effect of various x-radiation exposures on the sensory responses of the squid embryo and larva as evidenced by the tropistic responses and chromatophores.

MATERIALS AND METHOD

The squid eggs and early embryos were procured by the Supply Department of the Marine Biological Laboratory in the form of clusters of long strings, and were brought immediately to the laboratory where they were kept in running sea water. In general, all eggs or embryos in a single "finger" of the string mass are in the same stage of development. This is less apt to be the case as development progresses, because the effect of the slight differential in oxygen supply will become increasingly evident. The stages used in this series were, however, early (prehatching) and were essentially similar in respect to the stage of development for each set of observations.

The embryos[2] were exposed to the radiations while still within their capsules and jelly strings. They were placed in 40 cc. of fresh sea water in celluloid containers. These were circular De Witt Bait boxes, called Pyrashell, measuring 65 mm. in diameter and 16 mm. deep. The controls were kept under identical conditions during the irradiation of the experimentals. Immediately after irradiation, the embryos (both experimentals and controls) were transferred to regular finger bowls, each of which contained 100 cc. of fresh sea water, and suspended in a water bath kept at a constant temperature of 20° C.

The x-radiation conditions were as follows. The radiation equipment used in this series was the standard equipment of the Marine Biological Laboratory, Woods Hole, Mass. This consists of a pair of General Electric Coolidge tubes, water cooled, operated at 185 kvp and at 30 ma. At the low exposures one tube was

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The author acknowledges, with appreciation, the technical assistance of Mrs. Lona Barthelmes.

[2] For convenience the term "embryo" refers to the pre-hatching and "larva" to the post-hatching stage, regardless of how hatching is achieved.

Plate I

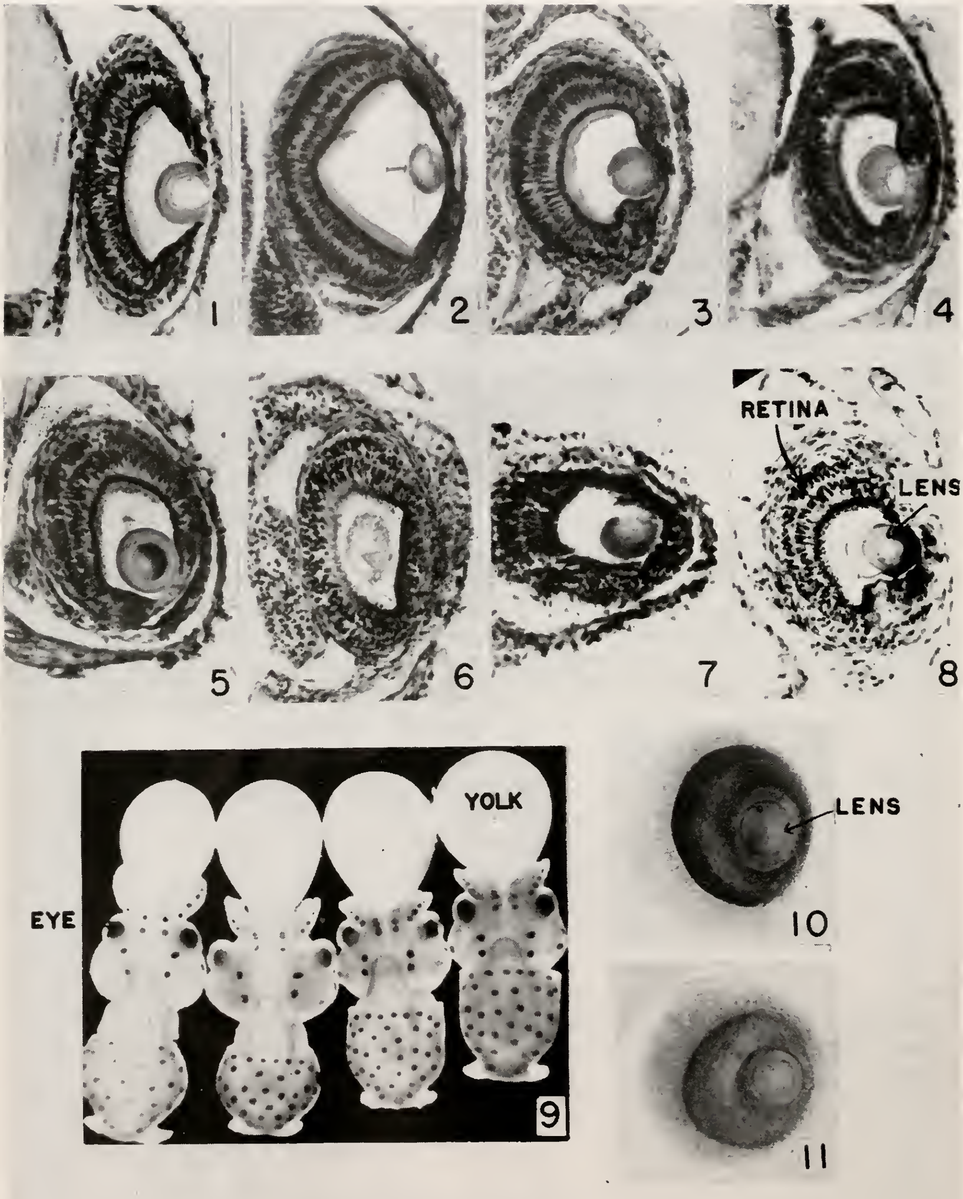
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PLATE I

blocked out and the distance was such that 30 roentgens were delivered to the specimens per minute. For the doses above 1000 r the two tubes were used, without filters, at such a distance that the dose rate was 2720 r per minute. X-ray exposures ranged from 50 r to 200,000 r in single exposures.

Observations and Experimental Data

There were three immediate and pronounced physiological effects of high dosage x-ray exposure of the early squid embryo, namely a loss of the positive phototropism and the negative geotropism, and the rupture of the jelly capsule, liberating the embryo.

Tropisms. Prior to x-irradiation, all hatched squid embryos will swim upward (negative geotropism) and toward any source of light (positive phototropism). These inevitable reactions are present for the unhatched but motile embryos as can be demonstrated by artificially (surgically) liberating them from their capsules. The phototropism is first manifested when the eyes become structurally differentiated, and the negative geotropism becomes evident when the embryonic siphon begins to function, causing the embryo to move vigorously. The weight of the embryo, with its large yolk sac (Fig. 9), normally causes it to sink, except that this negative geotropistic response tends to keep the embryo bobbing up toward the surface where, no doubt, it acquires a more abundant supply of oxygen.

Low dosage x-irradiation has very little effect on the tropistic responses. Only when the exposures reach 10,000 r or more is there any obvious (statistical) variation in responses. At this dosage level less than 50 per cent of the free-swimming larvae will move toward the light. At exposures of from 1000 r to 5000 r only stray larvae were seen swimming about aimlessly, with no evidence of tropistic direction. Not until 100,000 r is reached do the embryos appear totally indifferent to any source of light.

Histologically one cannot find any immediate change in the retina or lens which might be correlated with the functional tropistic changes. The radiation effect is

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Plate I

Figures 1–11

Figure 1. Section through eye of control squid embryo at same age as Figures 2 through 19, and identical with left specimen in Figure 9. (Compare with other control photographs, Figures 12 and 13.)

Figure 2. Section through eye of squid embryo four days after the embryo received 50 r x-rays.

Figure 3. Same as Figure 2 except exposed to 100 r x-rays.

Figure 4. Same as Figure 2 except exposed to 200 r x-rays.

Figure 5. Same as Figure 2 except exposed to 400 r x-rays.

Figure 6. Same as Figure 2 except exposed to 800 r x-rays.

Figure 7. Same as Figure 2 except exposed to 20,000 r x-rays.

Figure 8. Same as Figure 2 except exposed to 50,000 r x-rays.

Figure 9. Four squid embryos prior to hatching, three of which were exposed four days previously to x-irradiation. From left to right: Control, 1000 r, 5000 r, 50,000 r. Note persistence of embryonic chromatophores and yolk mass, and stunting of the irradiated specimens.

Figure 10. Entire eye of control (from Figure 9) showing large normal lens.

Figure 11. Entire eye of squid embryo which received 100,000 r x-rays four days previously. Entire eye small, with retina and lens smaller than control.

Plate II

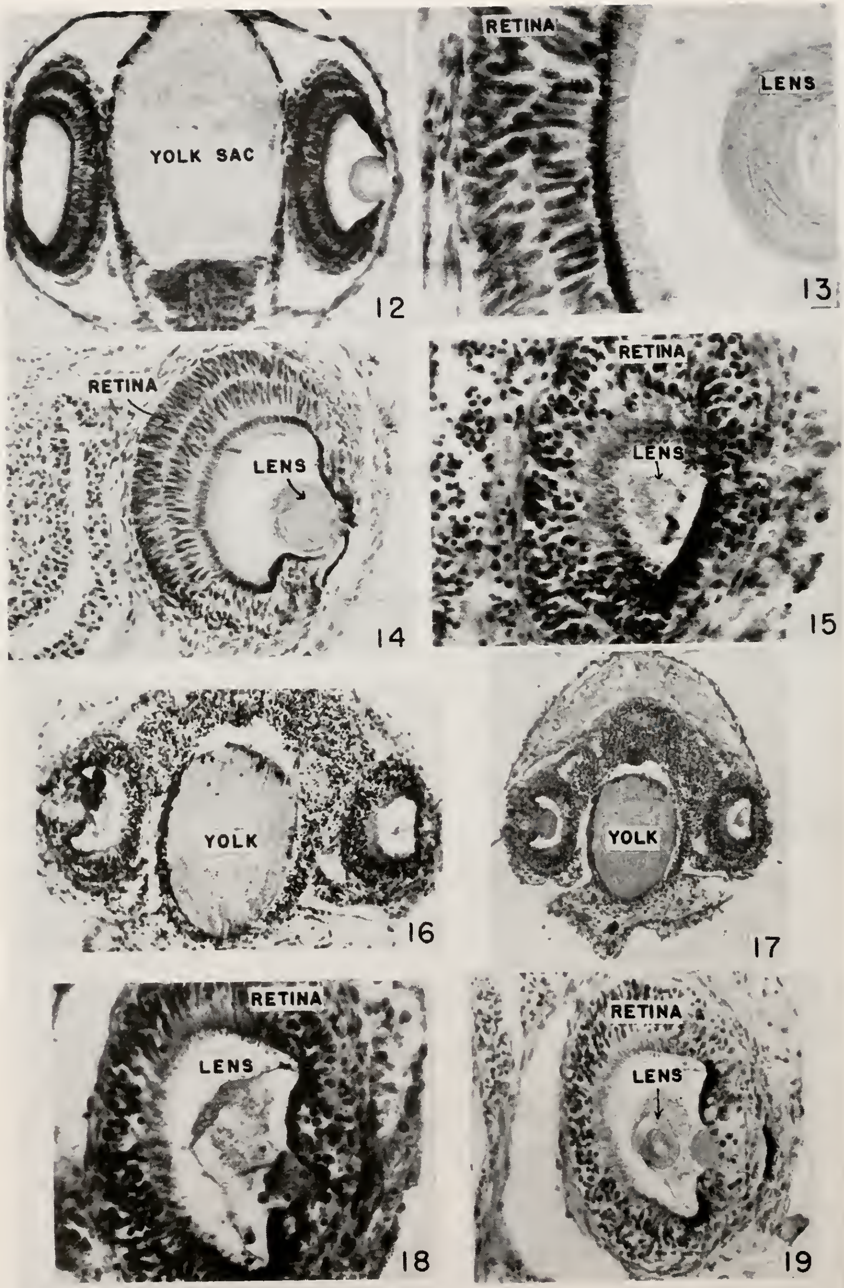
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PLATE 2

immediate upon the behavior of the embryo but is delayed with respect to observed histological changes in the optic system, as discussed below.

The negative geotropistic response is probably due to the nervous system as a whole, not particularly to the optic system. It is not so easily altered. By 50,000 r the embryos swim about aimlessly (with regard to the gravitational field) settling on the bottom of the container where their expected survival is poor because, presumably, there is insufficient oxygen to maintain the ontogenetic changes that are ensuing. Since, in contrast with the eye as the seat of phototropic responses, there is no known sensory center for geotropistic responses, one can only analyze the behavior of the embryo with respect to the forces of gravity.

High dose x-irradiations do not immediately kill the early embryo of the squid even though the tropistic responses are altered. Some embryos survive 200,000 r for as much as 120 hours.

Chromatophores. The chromatophores of the early squid embryo are few in number but they are very large, relative to the size of the embryo as a whole (Fig. 9). They are also very active, long before the time of hatching. There is no experimental evidence as to their control as there is for fish chromatophores (Parker, 1940; Tavolga and Rugh, 1947; and Tavolga, 1949). X-irradiation at any level used (50 r to 200,000 r) had no effect on the contractility of the chromatophores although it slightly altered the rate, accelerating their contractions and expansions. It did, however, stop the growth of the chromatophores as of the time of high dose x-irradiation, so that such embryos remained small and relatively dark as compared with the controls which grew larger, and paler. This effect is readily apparent after 3 or 4 days when there is sufficient ontogenetic change in the controls with which to compare the x-irradiated embryos (Fig. 9). The persistence of the functioning embryonic chromatophores following x-irradiation has been demonstrated for other embryos (Rugh, 1949). The chromatophores continue to function, so that the neuromuscular corollaries are unaltered, as long as the embryo is alive. The elements which control the chromatophores and the tropistic responses are therefore probably different.

PLATE II

FIGURES 12–19

(Note: Figures 12, 16, 17 at same magnification. Figures 14 and 19 at same magnification. Figures 15 and 18 at same magnification. Figure 13 at twice the magnification of 15 and 18.)

FIGURE 12. Section through bilateral eyes of squid embryo at termination of experiment, showing normal relation of optic cup, lens, and "corneal" epithelium. Compare with Figures 16 and 17.

FIGURE 13. High power magnification of Figure 12 control, showing highly developed retina and lens of the squid.

FIGURE 14. Squid embryo exposed to 100 r x-rays, showing slight eye damage after 4 days. General topography of the eye parts unchanged, but epithelium slightly irregular.

FIGURE 15. Disorganized retina and lens 4 days following x-irradiation to 800 r.

FIGURE 16. Section through bilateral eyes of living and functional squid embryo 4 days following x-irradiation to 20,000 r. Note extensive necrosis.

FIGURE 17. Same as Figure 16 except x-irradiated to 50,000 r. Note retardation in development (size).

FIGURE 18. Disorganized retina and lens 4 days after exposure to 50,000 r x-rays. Note many pycnotic nuclei.

FIGURE 19. Same as Figure 18 except that embryo was exposed to 100,000 r x-rays.

Hatching. The hatching process of the squid embryo requires the rupture of the thick jelly capsule and the subsequent emergence of the free-swimming embryo or larva. Normally this hatching process is due to the elaboration of a hatching enzyme, as in the case of fish (Armstrong, 1938) and the amphibia. At low x-ray exposures (i.e., 50 r to 1000 r) there is little induction of hatching, but as the dose approaches 50,000 r nearly 100 per cent of all embryos will be artificially hatched, even though they may not have been developmentally prepared for it. This premature hatching is due to a swelling and bursting of the jelly membrane around the embryo. This swelling of the jelly membrane, due probably to imbibition, appears far in excess of any parallel change in the embryo itself, possibly because of the high water content of the jelly. This phenomenon of the capsule was demonstrated by Evans, Beams and Smith in 1941 for the Arbacia egg. It is most graphically seen around the very early egg (i.e. cleavage stage) when the space between the membrane and the egg is almost doubled by high exposure to x-irradiation. Such eggs are, of course, immotile and are not generally liberated as are the active embryos of later development.

Muscular Activity. The early squid embryo consists of a large yolk sac which is surrounded by a thin muscular membrane (envelope) connected with the differentiating organs of the embryo proper (Fig. 9). As the yolk is consumed the embryo grows until the reduced yolk sac is gradually surrounded by the growing squid tissues. All during this process there is a constant, rhythmic contraction of the muscular envelope surrounding the yolk. These contractions vary only with the general environmental temperature and the activity of the embryo. When exposed to high doses of x-radiation there is only a slight acceleration of the contraction rate. Even at the very high exposures of 200,000 r these rhythmic smooth muscle contractions were not particularly affected, and were seen to persist long after the loss of the tropistic responses and until just before the embryo succumbed.

Histological Effects. There is always a latent period in the histological manifestation of x-irradiation damage (Rugh, 1949). The squid embryo is no exception, but since it differentiates rather slowly, it is not until 72 to 120 hours after exposure that one can observe significant changes in the retina and lens of the eye, for instance. All tissues are affected, but the greatest damage appears to be on the optico-ocular system and since this is presumed to be the system responsible for the phototropism, our description will be limited to the retina and the lens.

As previously stated, the eye of the squid is morphologically far advanced for an invertebrate organism and its visual acuity is evident from a period long before hatching (Figs. 1, 12, 13). The histological corollaries of loss of tropistic reactions are not evident for 3 to 4 days after x-irradiation at which time the retina of the 100 r or 200 r organism shows only slight disorganization (Figs. 14, 4), and the retina of the 800 r or more organisms shows extensive disorganization (Figs. 6–8, 15–19). The nuclei and cells of the control retina are arranged in concentric layers. While the x-irradiated eye at 800 r (or more) retains its spherical shape (Figs. 10, 11) the retinal layers lose their regularity and the lens becomes aspherical, and its fibers become disarranged (Figs. 15–19). There is no growth following x-irradiation so that the eye mass remains small (Figs. 10, 11). The histological changes can be seen readily by comparing Figures 1 through 8, all of which were magnified to the same degree, and all were from specimens fixed 4 days following x-irradiation of the experimentals. All were alive and active at the time of fixation. The extensive necrosis and pycnosis, even at 50,000 r and 100,000 r, were apparently not sufficient to kill the organisms (Figs. 18, 19).

SUMMARY AND CONCLUSIONS

1. The pre-hatched squid embryo can tolerate x-irradiation exposures to at least 200,000 r without succumbing for 4 days.

2. X-irradiation of the average squid embryo, within its jelly, to 25,000 r will liberate it from its capsule; while 50,000 r induces 100 per cent hatching. Irradiation-induced hatching is brought about by a swelling of the jelly and a bursting of the capsule, allowing the liberation of the motile larva.

3. The strong phototropistic response of all recently hatched squid larvae is immediately nullified in many specimens following x-irradiation to 10,000 r, but 100,000 r completely nullifies this response in all larvae, although they remain motile.

4. The strong negative geotropism of the squid larva is immediately reduced by 50,000 r and completely nullified by 100,000 r. This effect is also immediate, but the larvae remain motile.

5. Growth of squid larvae is stopped immediately by x-irradiation to 5,000 r or more, but no dose used (up to 200,000 r) had any immediate effect on the functioning of the smooth muscles, so that the motility of the organism as a whole was unaffected. The chromatophores and eyes remained the size they were at the time of x-irradiation.

6. The histological effects of x-irradiation were most damaging to the retina and lens of the eye, but were not manifest for 3 to 4 days. The retina showed extensive disorganization within 4 days following 800 r (or more) but since larvae lost their tropistic responses immediately (following high exposures) it must be postulated that long before these latent histological effects were evident, the retina was functionally disconnected from the motor organs which directed the normal organism toward the light source.

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THE ELECTROCARDIOGRAM OF ARENICOLA

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There is evidence, on the basis of the accelerating action of acetylcholine, that the hearts of Arenicola and Lumbricus are neurogenic (Prosser and Zimmerman, 1943). Nerve cells, which may be pacemakers, are present in the hearts of Arenicola (Carlson, 1908). Another test of the nature of the pacemaker is the electrocardiogram (ECG) which in myogenic hearts (vertebrates and molluscs) consists of regular slow potentials and in neurogenic hearts (Limulus, decapod Crustacea, some insects) is oscillatory. The electrocardiogram of arthropods may, in addition, have slow components but the oscillations appear to correspond with the repetitive discharge of the pacemaker neurons (see Prosser *et al.*, 1950, for references). The electrocardiographic test of the pacemaker has not been applied to annelids and, in fact, no electrocardiograms of annelids have been published.

Electrocardiograms of Arenicola were recorded with an Offner amplifier and crystograph; the amplifier was capacity coupled and of variable time constants, the longest being 0.2 seconds. Electrodes were of silver-silver chloride either in direct contact with the tissue or connected by cotton wicks soaked in sea water. Efforts to record electrocardiograms from Lumbricus and Neanthes were unsuccessful but extensive records were obtained from the hearts of six large specimens of *Arenicola cristata.* The worms were anesthetized with chloretone, the two hearts were exposed and recovery from the anesthesia was permitted in running sea water as described by Prosser and Zimmerman (1943). One worm was pinned to a paraffin block and the hearts exposed without anesthesia, but excessive body movement resulted in an unsatisfactory preparation. Usually an indifferent electrode was placed on the liver or inner body wall and an active electrode was put on different parts of a heart, from the lateral gastric vessel down to the ventral end. There was much interference with electrocardiograms by slow potentials arising from movements of the intestine and muscles of the body wall, and it was necessary to make many records from each animal with varying electrode positions, several time constants and different states of recovery from anesthesia to obtain a consistent picture of the electrocardiogram.

Several typical records are shown in Figure 1. The electrocardiogram (ECG) of *Arenicola cristata* consists of an initial rapid negative wave followed by a positive deflection, often with several superimposed oscillations (1a, 1b, 1c, 1e, 1f). The number, duration and shape of the oscillations are variable and they usually terminate with a sharp negative spike (1c, 1e) which may be followed by a slow negative wave (1d, 1f). The precise form varies in the same locus from time to time and from one region to another; such variability is more characteristic of oscillatory than of slow-wave types of electrocardiogram. If slow potentials had been present, the amplifier would have detected them as shown by the slow potentials recorded from hearts of squid and mussel (Fig. 2) and the many interfering slow potentials from body wall and intestinal muscle.

In Arenicola the beat normally starts in the lateral gastric vessel and spreads ventrally over the heart. Records taken from the dorsal portion (lateral gastric vessel) and from middle and ventral regions were qualitatively similar and showed no greater differences than did records from the same region at different times during a two-hour period of observation. Similar records were obtained whether wicks or wires made contact and electrode movement was so slight that it did not introduce an artifact. Regular electrocardiograms were occasionally observed when there was no visible contraction.

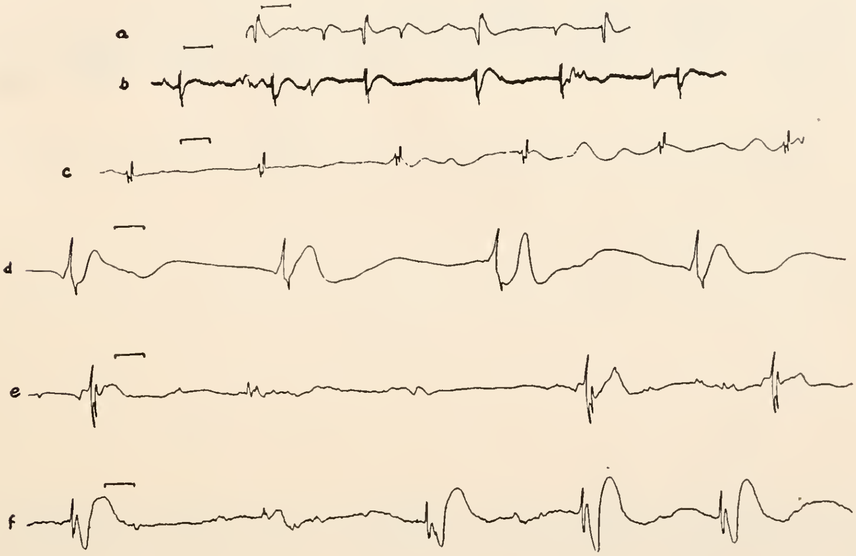

FIGURE 1. Electrocardiograms from Arenicola. Monopolar recording. In all records except 1a an upward deflection indicates negativity of the heart lead. 1a and 1b from same animal, all others different individuals. Time mark 1 second. In 1a three downward deflections between electrocardiograms were caused by activity of somatic musculature. Long time constant for records 1d and 1f, short time constants for other records. Note oscillatory spikes in 1a, 1b, 1c and 1f.

It was not possible to obtain a mechanical record of the contraction simultaneously with the electrical record. However, the heart was closely observed and the dilation and systole marked on the moving recording paper. The fast, initial component of the ECG consistently occurred before or simultaneously with dilation and the systole occurred later, often during the slow wave. Whether the fast potential is an action potential associated with active dilation, or whether there is a comparatively long delay between the action potential and systolic contraction is not certain, but the latter alternative seems more likely.

For comparison with Arenicola the electrocardiograms of known neurogenic hearts (Limulus and the spider crab Libinia) and known myogenic hearts (Loligo and a fresh-water mussel Amblema) are given in Figure 2. The records from Limulus (Fig. 2a, b) resemble string galvanometer records (Garrey, 1932) and the one from Libinia (Fig. 2c) resembles records from Astacus (Hoffman, 1911), while the molluscan electrocardiograms (Figs. 2d, e, f) resemble records from Octopus (Luisada, 1932) and Ostrea (Taylor and Walzl, 1941). The electrocardiograms of Arenicola do not have the persistent oscillations of the arthropods but they do show several variable spikes and lack the large regular slow waves of molluscs and vertebrates. In one preparation a long series of spikes appeared without any visible beat; such irregular spikes are sometimes found in records from Limulus hearts.

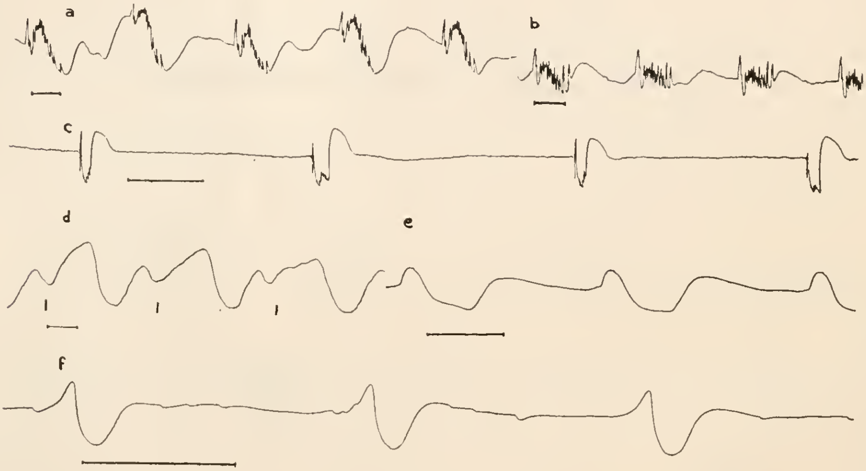

FIGURE 2. Electrocardiograms from neurogenic and myogenic hearts. Monopolar recording, upward deflection indicates negativity of active lead. 2a, c, d, e long time constant, 2b short time constant. Time records 1 second. a, b, Limulus. c, spider crab Libinia. d, e, fresh-water mussel Amblema. f, gill heart of squid Loligo. In 2d three vertical lines indicate point of systole. Note oscillatory spikes in 2a, 2b, 2c and presence of slow waves only in 2d, e and f.

It appears, therefore, that the electrocardiogram of Arenicola consists of summated spike potentials which may correspond to discharges from a ganglionic pacemaker. The fast components may be followed by a slow potential, the nature of which is as uncertain as it is in arthropod hearts. The electrocardiogram supports the hypothesis that the heart of Arenicola is neurogenic.

SUMMARY

The electrocardiogram of *Arenicola cristata* consists of a rapid negative deflection followed by a positive deflection, often with superimposed oscillations and often followed by a slow negative wave. There is much variability from animal to animal and from time to time at the same position.

The oscillatory nature of the electrocardiogram supports the hypothesis that the heart of Arenicola is neurogenic.

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DUALITY IN THE ELECTRICAL RESPONSE OF THE LATERAL EYE OF LIMULUS POLYPHEMUS

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Photoreceptor activity is accompanied by electrical changes, and the lateral eye of Limulus is no exception. Illumination of this photoreceptor produces a transient change in voltage (Graham, 1932; Hartline, 1928; Hartline and Graham, 1932). This change in voltage, called the retinal action potential, is measurable across the eye, front to back, and its polarity is such that the corneal surface becomes transiently negative to the back of the eye.

The retinal action potential recorded from the lateral eye of Limulus in response to a short period of illumination (Graham, 1932) exhibits a rather simple contour, consisting of a monophasic negative wave which returns to the base line. Close inspection of this response (Fig. 2, Graham, 1932) reveals a small step-like initial elevation which precedes in time the larger negative wave. Heretofore, no study, nor even any mention, has been made of this initial phase of the retinal action potential of the lateral eye of Limulus. The experiments which are the subject of this report describe some of the properties of this initial phase and indicate its possible source.

Materials and Methods

A rectangular piece of the carapace, with the lateral eye in the center, was cut out and dissected free from the underlying tissue. This section of the carapace was mounted in a lucite electrode chamber with a low melting point wax, which also covered all of the outer surface of the carapace, except the cornea. The corneal surface of the eye was bathed by sea water in the forward compartment, in the bottom of which there was a silver-silver chloride electrode. The back of the eye was bathed by sea water in the rear compartment which also contained a silver-silver chloride electrode. The chamber was then covered and placed in the path of a light beam in an otherwise light-tight compartment, and the silver-silver chloride electrodes were connected to the grids of the amplifier. One hour in the dark was found to be sufficient for complete dark-adaptation.

The light source was a 500 watt tungsten filament projection bulb. The light beam passed through 8 cm. of water and a heat absorbing filter. The intensity of the light beam was controlled by Wratten neutral tint filters. The maximum intensity incident on the cornea was 27,000 foot candles, which was considered as unit intensity. The duration (0.01 sec.) of the light flash was controlled by a photographic shutter. A portion of the light beam activated a photocell, the output of which was recorded as the stimulus signal. The potentials were fed into high-gain, condenser-coupled amplifiers and recorded on an ink writer. The frequency response of the ink-writer is flat to 100 cps. The amplifiers were operated at different time constants, in order to accentuate the duality of the response. The nominal time constants were calibrated with a square wave voltage pulse.

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[1] Lalor Fellow, Summer, 1949, during which period a large part of the work reported was completed.

After dark adaptation of the excised eye, a flash of light of the lowest intensity was admitted and the response recorded. Ten minutes later a second flash of light was admitted and so on until the desired intensity range was covered. When a second series was desirable, an hour was again permitted for completion of dark adaptation and the sequence was repeated.

The effect of light-adaptation upon the electrical response of the excised eye was determined by admitting a test flash of light 5 seconds after the end of a controlled period of exposure to light. The intensity of and exposure to the adapting light could be readily varied.

A third experimental procedure consisted of the introduction of substances into the sea water bathing the eye. These materials were introduced under dim ruby-red light. Recovery from the exposure to red light was permitted from 2 to 7

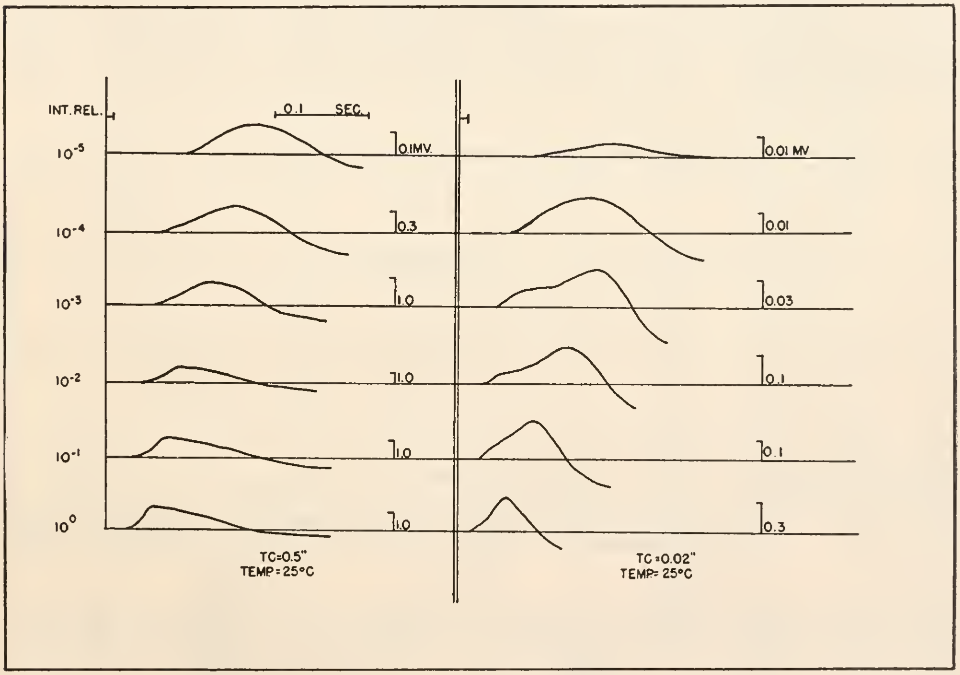

FIGURE 1. Protocols of typical experiments with the lateral eye of Limulus. The original records were reenforced with black ink for photographic reproduction.

Left. A series of responses elicited by test flashes of 0.01" duration (indicated in the upper line only) and of intensities (10° represents 27,000 foot candles) indicated in the left hand column (int. rel.) opposite the appropriate records. These responses were recorded with 0.5" time constant. Upward deflection indicates negativity of the corneal electrode. Calibration signal (millivolts) follows each record. The time base is indicated above the upper record.

Right. A similar series of responses recorded with a short time constant (0.02") to emphasize the dual nature of the response. Exposure, intensity, calibration and time base as above.

minutes, depending upon the intensity of the next test flash. In control experiments, the exposure to red light did not affect the magnitude or contour of the response to a test flash administered immediately after the exposure to the red light, presumably because the intensity of the test flash was sufficiently high.

Forty-eight experiments were conducted at room temperature, which varied from experiment to experiment in the range 25° to 28° C. The crystograph pens recorded with red ink, which had to be reenforced with black india ink for photographic reproduction.

Results

1. Results obtained with 0.5″ time constant

The responses recorded from an excised Limulus lateral eye, stimulated with light flashes of different intensities, exhibit a rather uniform contour and a progressive increase in magnitude (a typical series is illustrated in Fig. 1, left). The relation of retinal action potential magnitude (measured from base line to peak of the negative wave) to the common logarithm of the stimulating intensity (Fig. 2) indicates a tendency to level-off at low and high intensities, with a rather linear intermediate portion. The latency of the response decreases with increasing intensity (Fig. 2, crosses).

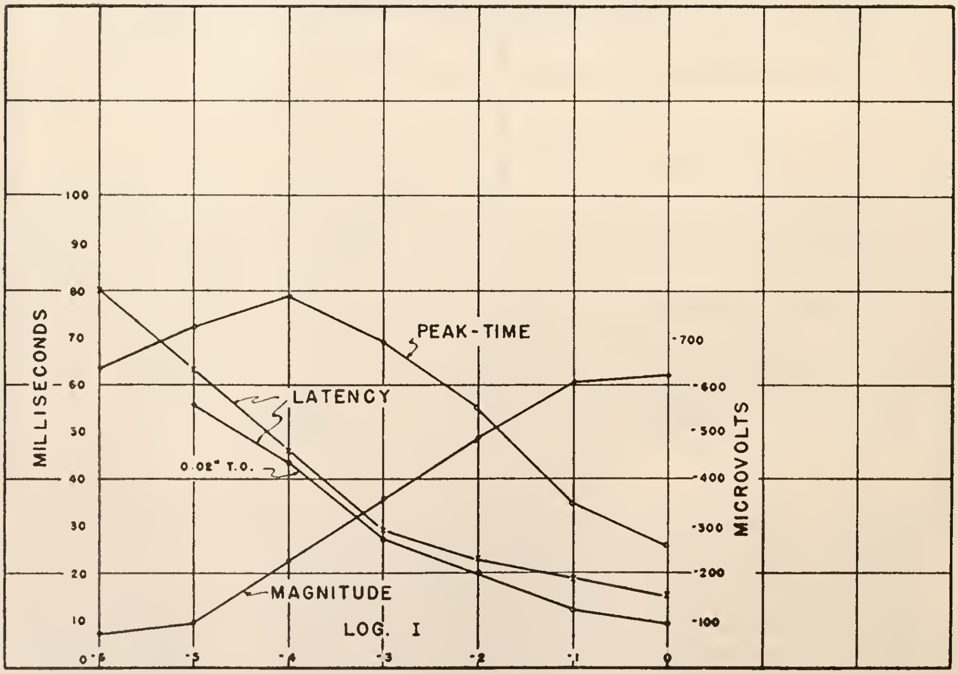

Figure 2. Graphs relating latency, peak-time and magnitude of the retinal electric response to the logarithm (base 10) of the stimulating light intensity. Log 0 represents an intensity of 27,000 foot candles. All data were obtained from responses recorded with 0.5″ time constant, except latency curve marked 0.02″ T. C.

2. *Results obtained with 0.02″ time constant*

These responses were obtained in a manner similar to the above and a typical series from one experiment is illustrated in Figure 1, right. The presence of two phases in the electrical response to flash intensities of 1 × 10^{-3} and greater is apparent. From such records and others not shown, in which the resolution of the two phases was even greater (Fig. 4, left, lower line), it was possible to measure the magnitudes of the two phases. The relation of these magnitudes to the common logarithm of the flash intensity is shown in Figure 3 (1, magnitude of the first phase; 2, magnitude of the second phase). The departure of this magnitude contour from the more conventional one of Figure 2 may be attributed to the short time constant, as discussed below.

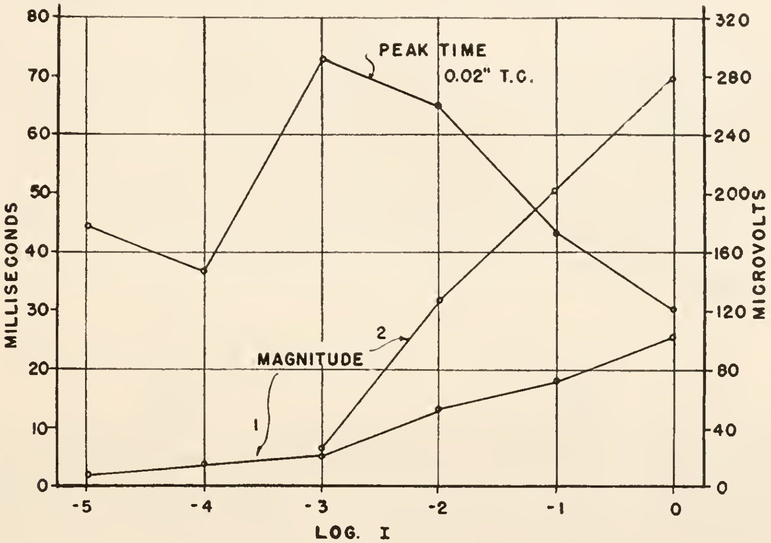

FIGURE 3. Graphs relating peak-time and magnitude of the retinal electric response to the common logarithm of the stimulating light intensity. These data are from responses recorded with a short (0.02″) time constant. The magnitude relation marked 1 pertains to the initial phase; that marked 2 pertains to the second phase of the retinal response.

The responses to flash intensities of 1 × 10^{-4} and 1 × 10^{-5} (Fig. 1, right) are of uniform contour and do not indicate two phases. The gradual regression, in time, of the second phase (Fig. 1, right) from the lower record on up, suggests another measurement; namely, the time required for the response to reach maximum, hereafter called peak-time. Peak-time is measured from the beginning of the response to its maximum. The relation of peak-time to the common logarithm of the intensity is illustrated in Figure 3 (short time constant) and in Figure 2 (longer time constant).

The latency of the initial phase of the retinal action potential decreases progressively with increasing intensity, giving the relation shown in Figure 2 (0.02″ T. C.).

3. Effect of light adaptation

The effect of light adaptation on the retinal electric response, using the short time constant, is illustrated in Figure 4, left. Within the particular conditions of this experiment, it is apparent that light adaptation of 200 foot-candle seconds renders the initial phase invisible and causes some reduction of the magnitude of the second phase. These effects are quite reversible, as indicated in the control record, obtained 10 minutes after the preceding rsponse.

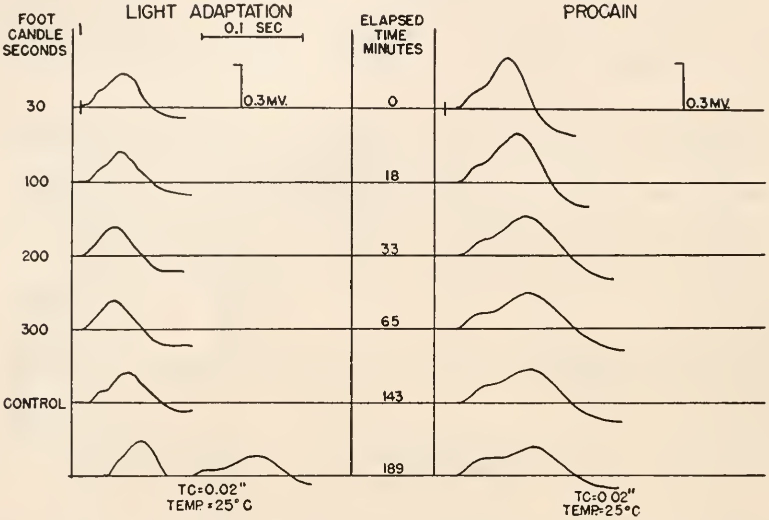

FIGURE 4. Protocols of experiments illustrating the effect of light adaptation (left) and procain (right) on the retinal electric response of the Limulus lateral eye elicited by a constant intensity, constant duration test flash (270 f. c., 0.01″). Column marked "foot candle seconds" indicates the exposure to which the eye was subjected preceding the test flash, which was administered 5 seconds after the adapting light was cut off. Column marked "elapsed time minutes" indicates time elapsed (eye in dark) after the drug was administered. Lower line of left column illustrates the extremes of dissociation of the two phases encountered during these experiments. Amplification and paper speed were constant throughout and are indicated on upper line.

4. The effect of procain hydrochloride

The addition of procain hydrochloride to the sea water in the rear compartment of the eye chamber, in amounts to bring the final concentration to 0.02 per cent, produced immediate cessation of optic nerve activity and effected a gradual reduction in the magnitude of the second phase of the retinal response (Fig. 4, right). The magnitude of the initial phase is but little affected, even in responses (not shown) recorded six hours after introduction of the drug.

5. Variability of dissociation of the two phases

The extent to which the two phases of the retinal action potential are summated varies somewhat from preparation to preparation (Fig. 4, left column, lowest line).

The dissociation of the phases in any one preparation was rather constant throughout. The presence or absence of the rudimentary eye (the mass of white pigment behind the lateral eye) did not affect the two phases of the retinal action potential in any way.

Discussion and Conclusions

1. *Is the double response an artifact?*

a) Excised lateral eye preparations kept for 8 hours at room temperature generally showed the onset of deterioration by a gradual reduction in magnitude of response. Both phases of the retinal action potential were affected. Preparations kept at room temperature for 20 hours often were totally unresponsive. Apparently both the initial and the second phase are characteristic of living eyes.

b) The dual response has been recorded with silver-silver chloride electrodes and with salt bridges leading to calomel half-cells. The dual response is also apparent in published records (Fig. 2 of Graham, 1932) obtained with an entirely different amplifying and recording system. The duality of the electrical response cannot be considered an artifact attributable to the recording electrode system.

c) The initial phase disappears upon light adaptation (Fig. 4, left) and reappears upon dark adaptation. The initial phase appears in time after a characteristic latency which varies with the intensity of the incident illumination (Fig. 2). These facts indicate that the duality of the retinal action potential is characteristic of the functional photoreceptor.

2. *Which phase persists at low intensities?*

Measurements of the time from the onset to the crest of the retinal action potential (peak-time) plotted as a function of the log intensity (Fig. 3) reveal a marked discontinuity between $\log I = -3$ and $\log I = -4$. This indicates that the crest of the response to a flash intensity of 10^{-4} occurs earlier in time than the crest of the response to a flash intensity of 10^{-3}, which is apparent also in Figure 1, right column. Scanning of these records from bottom to top indicates that the crests of the responses to flash intensities of 10^{0} to 10^{-3} are the crests of the second phase. At 10^{-4} only one phase is present and, since the crest occurs earlier in time, it is implied that this is the crest of the initial phase. The second phase is apparently absent at this and lower intensities.

A similar conclusion follows from consideration of the magnitude-log intensity relationships of Figure 3. The magnitude of the second phase (2) decreases rapidly from 10^{0} to 10^{-3}. Extrapolation of this curve would indicate a complete absence of the second phase in response to flash intensities of 10^{-4} or lower. It must be suggested, however, that recording with a short time constant sacrifices a large part of the transient voltage change, particularly that associated with the second phase. This undoubtedly produces some distortion of the magnitude-log I relation (compare with a similar curve, Fig. 2). The initial phase, on the other hand, shows a gradual increase in magnitude and the maximum at $\log I = 0$ is appreciably less than the magnitude of the second phase. Such data were obtained from six experiments where the degree of dissociation was sufficient to make these measurements possible throughout the entire intensity range.

3. *The significance of the two phases*

The rapid disappearance of the initial phase upon light adaptation, coupled with the fact that low intensity light flashes elicit only the initial phase, suggests that this response may be associated with receptor cells of greater sensitivity than those which give rise to the second phase. This consideration implies that the lateral eye of Limulus contains two functionally different kinds of sense cells. It is of great interest that two structurally different kinds of sense cells have been described for the lateral eye of Limulus (Demoll, 1914). The complex eye of Limulus is comprised of many visual units, the ommatidia. All ommatidia, according to Demoll (1914), possess a similar spatial arrangement of receptor cells (Fig. 5). There are a group (10–15) of receptor cells radially arranged about the longitudinal axis of the ommatidium, leaving a hollow core in the center. This core is partially occluded by a process originating from an eccentrically located sense cell (Fig. 5). In each ommatidium there is only one eccentric sense cell and this seldom contains pigment, whereas the radially located sense cells often contain pigment (Demoll,

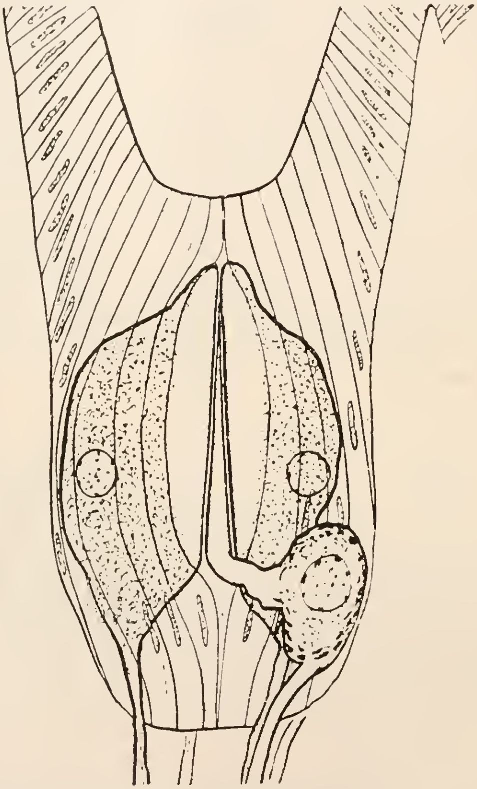

FIGURE 5. Diagrammatic representation of a longitudinal section through an ommatidium from the lateral eye of Limulus showing the radial retinal cells and the eccentric retinal cell with its central process, all embedded in an epithelial framework. (From Demoll, 1914.)

1914). The relative paucity of the eccentric sense cells, their centrally located processes and their freedom from pigment makes them the likely source of the initial phase of the retinal action potential. Further, in certain experiments the magnitude of the second phase of the retinal action potential has exceeded 1 millivolt, whereas the initial phase rarely exceeded 0.1 millivolt. This response magnitude ratio of 10 to 1 roughly parallels the population ratio of radially arranged sense cells to eccentric sense cells, 10—15 to 1. It is concluded, therefore, that the response of the eccentric sense cells to illumination produces the initial phase and the response of the radial sense cells produces the second phase of the retinal action potential.

The presence of two morphologically and physiologically different types of receptor elements in the lateral eye of Limulus is reminiscent of the dual nature of most vertebrate retinae.

SUMMARY

Retinal action potentials recorded from the excised lateral eyes of Limulus, using a short time constant amplifier coupling, show a dissociation into two discrete responses, an initial and a secondary phase, over part of the intensity range. Analysis of the data indicates that the single response present at low intensities of stimulation is the initial phase and that both phases are present only in response to higher intensities of illumination. This sequence of single and dual phases with increasing intensity of stimulation gives rise to the peculiar peak-time relation shown in Figure 3. Light adaptation causes a rapid disappearance of the initial phase and only a gradual disappearance of the second phase. The magnitude of the initial phase is much smaller than the magnitude of the second phase. These data suggest that the initial phase represents the response of highly sensitive receptors (eccentric retinula cells) that are outnumbered by less sensitive receptors which contribute to the second phase (the radial retinula cells) (Demoll, 1914). Procain hydrochloride administered to the excised eye results in a reduction in magnitude of phase 2, and little or no change in magnitude of phase 1.

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STUDIES ON THE ANAEROBIC METABOLISM AND THE AEROBIC CARBOHYDRATE CONSUMPTION OF SOME FRESH WATER SNAILS

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Field work attempting the eradication of schistosome-transmitting snails in various parts of the world has demonstrated the inadequacy of the hitherto available molluscacides. If control of schistosomiasis is to be attempted by means of chemical compounds interfering with the metabolic pathways of the intermediate hosts of the flukes, the establishment of a theoretical basis for further evaluation of potential and actual molluscacides is urgently needed.

In a previous paper by von Brand, Nolan, and Mann (1948), some data on the aerobic faculties of various species of snails are recorded. A detailed knowledge of the anaerobic metabolism of such snails is also necessary since, according to a personal communication by Dr. W. H. Wright, some schistosome-transmitting snails have the ability to escape the action of poisons by burrowing into the mud, which is usually very poor in oxygen.

In the present paper an attempt is made to answer some unsolved problems concerning the anaerobic metabolism of snails which, in fact, has so far never been studied. Specifically, the following points have been investigated: Anaerobic resistance of various species, the anaerobic carbohydrate consumption, carbon dioxide and lactic acid production, and the quantitative relationships between anaerobic and aerobic carbohydrate consumption.

MATERIAL AND METHODS

The following species of snails were employed and where no further information is provided they were of the same derivation as those used in a previous study (von Brand, Nolan, and Mann, 1948).

1. Pulmonates

    PLANORBIDAE: *Australorbis glabratus; Helisoma duryi; Helisoma trivolvis* specimens collected near Brownsville, Texas; *Tropicorbis obstructus; Tropicorbis donbilli; Planorbarius corneus,* laboratory-reared; *Biomphalaria boissyi,* laboratory-reared from Egyptian specimens; *Biomphalaria pfeifferi,* laboratory-reared from Liberian stock.

    LYMNAEIDAE: *Lymnaea stagnalis* and *Lymnaea palustris,* both laboratory-reared from Douglas Lake, Michigan stock; *Lymnaea natalensis,* laboratory-reared from Dharan, Saudi Arabia, specimens.

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[1] The authors wish to express their appreciation for the contribution of snails to Dr. E. G. Berry, Mrs. M. O. Nolan, Dr. L. Olivier, and Mr. W. B. DeWitt of this Laboratory and Dr. H. van der Schalie and Mr. E. Abdel-Malek of the University of Michigan.

PHYSIDAE: *Physa gyrina; Physa cubensis,* laboratory-reared from Cuban stock; *Aplexa nitens,* laboratory-reared from specimens collected near Brownsville, Texas.

2. Operculates

AMNICOLIDAE: *Oncomelania nosophora,* laboratory-reared from Japanese stock.
POMATIOPSIDAE: *Pomatiopsis lapidaria.*
THIARIDAE: *Melanoides tuberculatus,* laboratory-reared from Dharan, ~~Saudi~~ Arabia, specimens.
PLEUROCERIDAE: *Goniobasis livescens,* used shortly after being shipped from northern Michigan.

All determinations were carried out at 30° C., a temperature corresponding approximately to the summer temperature in the Washington, D. C., area. It was well tolerated by all snails.

Warburg equipment was used throughout the work, flasks of about 17 and 5 ml. being employed for larger and smaller snails respectively. Two ml. of dechlorinated tapwater served as medium in the former, one ml. in the latter. Anaerobic conditions were established by passing a stream of 99.99 per cent pure Linde nitrogen, further purified by passing over heated copper, through the shaking flasks for 15 to 20 minutes.

In the aerobic experiments the previously described technique was again employed (von Brand, Nolan, and Mann, 1948).

The total carbohydrates were determined in the snail tissues by Dische and Popper's (1926) method, the color being read in a Fisher electrophotometer with a filter at 425 mμ, instead of in a visual colorimeter.

Barker and Summerson's (1941) method was used for the lactic acid determinations in the snail tissues and the medium.

RESULTS

1. *Anaerobic resistance*

Considerable differences in anaerobic resistance between various groups of snails occurred. The figures summarized in Table I show that the Lymnaeidae and Physidae withstood anaerobic conditions without apparent damage for only 6 hours, and that most died within 16 hours. The Planorbidae and the operculate snails were more resistant, most species surviving regularly for at least 24 hours, and some, especially *Helisoma trivolvis* and *Melanoides tuberculatus,* for 48 to 64 hours. *Australorbis glabratus* and *Biomphalaria pfeifferi,* however, were somewhat more sensitive, all specimens surviving regularly only 16 hours in the lack of oxygen.

The behavior of the snails under anaerobic conditions was quite characteristic. All extended maximally out of their shells and soon, at the latest within a few hours, became completely motionless. If not used for chemical determinations, the snails, after the end of the anoxic period, were placed into beakers containing aerated dechlorinated tapwater. As long as they were fully extended, they recovered completely, resuming motion soon after restoration of aerobic conditions. If the anaerobic period lasted too long, on the contrary, the snails began to hemorrhage and finally retracted into their shells. This seemingly indicates that the above-mentioned lack of motion was not a complete paralysis. Snails which had retracted into their shells during the anaerobic period did not recover during a subsequent aerobic period. Whether, in all cases, they actually died during the anaerobic period, or died shortly thereafter, could not be determined.

2. *Anaerobic metabolism*

A condensed summary of our experimental data (Table II) shows that, in general, two series were carried out with a given species of snails. In one series the CO_2 production and carbohydrate consumption were studied. In the second series the CO_2 production, the lactic acid content of the tissues, and the excretion of lactic acid into the medium were determined. The carbon dioxide determination then was common to both series. A survey of the figures shows that in pulmonates the CO_2 figures of both series were in most cases in reasonably close agreement, while considerably greater differences occurred in this respect in the two operculates *Pomatiopsis lapidaria* and *Melanoides tuberculatus*, so tested. No definite reason for the irregular behavior of these latter can be given.

TABLE I

Anaerobic resistance of various species of fresh water snails at 30° C.

Species	Period of anaerobiosis									
	6 hours		16 hours		24 hours		48 hours		64 hours	
	Number of specimens	Per cent surviving	Number of specimens	Per cent surviving	Number of specimens	Per cent surviving	Number of specimens	Per cent surviving	Number of specimens	Per cent surviving
Australorbis glabratus	72	100	59	98	12	25	12	0		
Helisoma duryi					36	100				
Helisoma trivolvis			6	100	6	100	6	100	5	100
Tropicorbis obstructus					110	100			12	25
Tropicorbis donbilli	12	100			12	66	6	0		
Planorbarius corneus	36	100			42	100			12	17
Biomphalaria boissyi	12	100	36	100	12	100	12	0		
Biomphalaria pfeifferi			67	100	12	50	12	0		
Lymnaea stagnalis	36	100	12	9						
Lymnaea palustris	36	100	12	0						
Lymnaea natalensis	48	100	12	0						
Physa gyrina	64	100	33	3						
Physa cubensis	72	100	6	0						
Aplexa nitens	36	97	12	0						
Oncomelania nosophora					30	100	18	17		
Pomatiopsis lapidaria					108	98	12	25		
Melanoides tuberculatus					60	100	12	92	12	81
Goniobasis livescens					12	100				

The length of the anaerobic period was chosen for each species in accordance with the resistance data discussed in the preceding section. In the cases of *Australorbis glabratus* and *Planorbarius corneus*, two periods of different duration were tested in order to see if significant differences in metabolism occurred. The shorter period corresponded to that used with the nonresistant species. The data (Tables II and III) gained from both periods agreed fairly well, with the exception of an unexplained larger excretion of lactic acid by Planorbarius during the shorter period. There is, therefore, no reason to assume that the differences in metabolism between resistant and nonresistant species mentioned below are correlated with the different lengths of anaerobic periods employed.

From the data summarized in Table II, the average hourly rate of metabolic processes has been calculated (Table III), and a perusal of both Tables II and III reveals the following facts.

Total carbohydrates. The highest carbohydrate content, 3.5 per cent of the fresh weight, was found in *Lymnaea natalensis,* while *Physa gyrina* showed the lowest, 1 per cent. The rates of anaerobic carbohydrate consumption of well-fed snails were highest in the Physidae and Lymnaeidae, intermediate in most Planorbidae, and lowest in operculates. The nutritional state of the snails was of considerable influence as is seen by the example of *Australorbis glabratus* which, after 2 weeks' starvation, consumed less than half the amount of endogenous carbohydrate catabolized by well-fed specimens.

TABLE II

Anaerobic metabolism of various species of snails

Species	No. of exper.[1]	Anaerobiosis	Entire exper. period. Carbon dioxide mm.^{3} per 1 gm.[1]	Carbohydrates in per cent		Lactic acid in tissues microgram per gm.		Lactic acid excreted microgram per gm.
		Hours		Initial	Final	Initial	Final	
Australorbis glabratus [2]	24; 24	16	641 ±20; 664 ±26	2.69 ±0.18	2.19 ±0.15	232 ±21	155 ± 29	422 ±121
Australorbis glabratus [2]	36; 36	6	187 ± 9; 203 ±14	2.58 ±0.19	2.47 ±0.15	150 ±22	150 ± 18	118 ± 25
Australorbis glabratus [3]	18	16	327 ±22	1.35 ±0.06	1.11 ±0.12			
Australorbis glabratus [4]	22	16	219 ±27	0.54 ±0.12	0.32 ±0.05			
Helisoma duryi	18; 18	24	819 ±36; 528 ±35	2.08 ±0.18	1.00 ±0.13	106 ±15	104 ± 19	114 ± 36
Tropicorbis obstructus	17; 18	24	1177 ±43; 1024 ±56	1.77 ±0.13	0.67 ±0.07	160 ±34	148 ± 21	357 ± 37
Planorbarius corneus	24; 17	24	760 ±44; 709 ±33	2.94 ±0.02	2.65 ±0.18	267 ±66	202 ± 38	131 ± 32
Planorbarius corneus	18; 24	6	141 ±12; 143 ±12	2.35 ±0.34	2.27 ±0.29	285 ±12	205 ± 11	252 ± 14
Biomphalaria boissyi	18; 17	16	646 ±35; 637 ±23	2.59 ±0.17	1.70 ±0.13	320 ±20	265 ± 24	692 ± 60
Biomphalaria pfeifferi	18; 18	16	752 ±45; 637 ±37	2.92 ±0.19	2.07 ±0.23	229 ±23	207 ± 32	506 ± 86
Lymnaea stagnalis	18; 18	6	479 ±34; 567 ±61	1.26 ±0.10	0.99 ±0.11	247 ±47	689 ±102	871 ±109
Lymnaea palustris	18; 18	6	300 ±34; 395 ±46	1.22 ±0.13	0.71 ±0.07	112 ±16	664 ± 87	34 ± 5
Lymnaea natalensis	18; 18	6	284 ±20; 237 ±24	3.55 ±0.17	3.25 ±0.10	302 ±48	756 ± 97	1000 ± 77
Physa gyrina	18; 17	6	144 ±11; 214 ±23	0.98 ±0.07	0.49 ±0.06	85 ± 9	313 ± 41	45 ± 9
Physa cubensis	17; 18	6	313 ±35; 395 ±46	1.22 ±0.11	0.76 ±0.08	185 ±34	590 ±161	63 ± 8
Aplexa nitens	17; 18	6	277 ±19; 371 ±21	2.05 ±0.10	0.94 ±0.11	185 ±17	384 ± 35	679 ± 66
Oncomelania nosophora	18	24	316 ±35	1.42 ±0.12	0.99 ±0.08			
Pomatiopsis lapidaria	12; 16	24	290 ±42; 646 ±89	1.47 ±0.08	1.05 ±0.08	114 ±25	98 ± 25	188 ± 45
Melanoides tuberculatus	18; 29	24	322 ±35; 177 ±24	2.73 ±0.13	2.37 ±0.13	89 ±20	128 ± 24	120 ± 34
Goniobasis livescens	12	24				97 ±13	93 ± 7	191 ± 24

[1] The first figures in these columns refer to the carbohydrate series; the second figures to the lactic acid series.

[2] Well-fed snails.

[3] Snails from an aquarium with scanty food supply.

[4] Snails starved for 2 weeks prior to the determinations.

The figures behind the plus and minus signs represent the standard error of the mean.

Lactic acid. The lactic acid content of the tissues of snails taken from aerated aquaria was relatively low, varying from about 9 mg. per cent in *Physa gyrina* or *Melanoides tuberculatus* to about 30 mg. per cent in *Lymnaea natalensis.* In contrast to these relatively small variations in preanaerobic lactic acid level, a very distinct difference in the anaerobic lactic acid levels was observed in different species. The Lymnaeidae and Physidae showed a marked accumulation of lactic acid within their tissues, while this did not occur in the Planorbidae and the operculate snails. In most species belonging to these latter groups the anaerobic level was even lower than the aerobic one. While most of these latter differences do not appear statistically significant, their repeated occurrence suggests a lactic acid excretion during anaerobiosis in excess of the amounts formed.

All species studied excreted lactic acid. The total production of lactic acid during the anaerobic period (lactic acid excreted plus lactic acid accumulated in the tissues, or lactic acid excreted minus lactic acid deficit in the tissues) was largest in Lymnaeidae and Physidae, but small in the other species.

TABLE III

Hourly anaerobic metabolic changes of various species of snails per 1 gm. tissue

Species	CO_2 mm.^{3} produced	Carbohydrate consumed, mg.	Lactic acid accumulated in tissues, mg.	Total lactic acid produced, mg.	Carbon balance per cent of total CO_2 liberated from bicarbonate by lactic acid	Per cent of consumed carbohydrate carbon accounted for by lactic acid
Australorbis glabratus [1]	41	0.31	0	0.022	13	7
Australorbis glabratus [2]	33	0.18	0	0.020	15	11
Australorbis glabratus [3]	20	0.15				
Australorbis glabratus [4]	14	0.14				
Helisoma duryi	28	0.45	0	0.005	4	1
Tropicorbis obstructus	46	0.46	0	0.014	8	3
Planorbarius corneus [5]	30	0.12	0	0.003	3	3
Planorbarius corneus [6]	24	0.13	0	0.029	29	22
Biomphalaria boissyi	40	0.56	0	0.040	25	7
Biomphalaria pfeifferi	43	0.53	0	0.030	17	6
Lymnaea stagnalis	87	0.45	0.074	0.219	63	49
Lymnaea palustris	58	0.85	0.092	0.098	41	12
Lymnaea natalensis	43	0.50	0.076	0.243	139	50
Physa gyrina	30	0.82	0.038	0.046	38	5
Physa cubensis	59	0.77	0.068	0.078	32	10
Aplexa nitens	54	1.85	0.033	0.146	67	8
Oncomelania nosophora	13	0.18				
Pomatiopsis lapidaria	20	0.18	0	0.007	9	4
Melanoides tuberculatus	10	0.15	0.002	0.007	17	5
Goniobasis livescens	9		0	0.008		

[1] Well-fed snails, 16 hours anaerobiosis.
[2] Well-fed snails, 6 hours anaerobiosis.
[3] Snails from an aquarium with scanty food supply.
[4] Snails starved for 2 weeks prior to the determinations.
[5] Well-fed snails, 24 hours anaerobiosis.
[6] Well-fed snails, 6 hours anaerobiosis.

The last column of Table III shows conclusively that lactic acid is, from a quantitative standpoint, a major end product of the anaerobic carbohydrate metabolism only in *Lymnaea stagnalis* and *Lymnaea natalensis*. In all other species, unidentified end products must predominate by far.

Carbon dioxide. The carbon dioxide excretion showed fairly large variations from species to species. Due to the calcareous shells it was, unfortunately, impossible to study the question of carbon dioxide retention in the tissues. Although the CO_2 figures are probably not absolutely correct, it is believed that the following conclusions are not far from the truth.

The next to the last column of Table III shows that in Lymnaeidae and Physidae a major portion of the carbon dioxide excreted was probably of inorganic origin, having been liberated from bicarbonate during glycolysis leading to lactic acid. In *Lymnaea natalensis* even more lactic acid was found than corresponded to the carbon dioxide excreted. In all other species the amount of CO_2 due to lactic acid was only moderate. In view of the absence of information concerning the other end products of the anaerobic carbohydrate metabolism, the question of whether or not the remaining CO_2 was also of inorganic origin or whether it represented true respiratory CO_2 must remain unanswered at the present time.

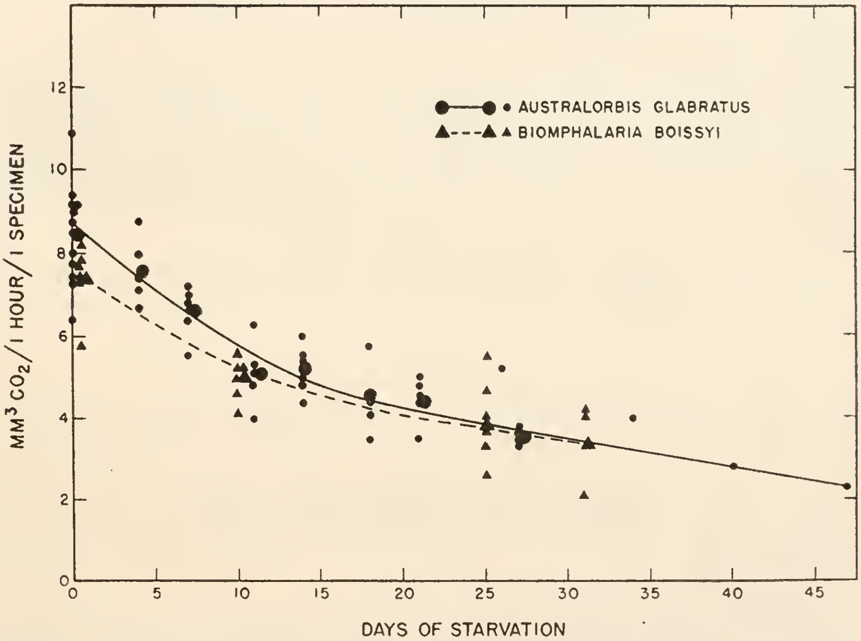

FIGURE 1. Influence of starvation on the anaerobic carbon dioxide production of aquatic pulmonate snails. Twelve well-fed specimens of *Australorbis glabratus* and 6 specimens of *Biomphalaria boissyi* were used. They were exposed on specified days to 6 hours anaerobiosis. Between the anaerobiosis periods the snails were kept starving in aerated, dechlorinated tap-water. The determinations were continued until the last snail of each group died. The figure shows the individual and the mean values (small and large symbols respectively).

During starvation (Fig. 1) the rate of anaerobic CO_2 production fell progressively but the decline was, on the whole, less pronounced than in the case of the previously studied aerobic metabolism (von Brand, Nolan, and Mann, 1948).

In several species the specimens used for the CO_2 determinations varied rather markedly in size. A study of this material (Fig. 2) shows that in intraspecific comparisons the CO_2 production followed the surface law rather well (relative surface calculated as weight ⅔), while the same obviously did not hold true for all cases of interspecific comparison.

3. *Aerobic carbohydrate metabolism*

The data summarized in Table IV demonstrate that all species consumed carbohydrate under aerobic conditions. The aerobic rate was always smaller than the anaerobic one as indicated by the ratio between anaerobic and aerobic rate given in the last column of Table IV. It is noteworthy, however, that the quotient was small in 5 out of the 7 species tested.

In *Planorbarius corneus* and *Pomatiopsis lapidaria* the amount of oxygen consumed by the snails was clearly in excess of the amount required for complete oxidation of the consumed carbohydrate. In the other cases it was either just sufficient to account theoretically for complete oxidation, or insufficient. It must be assumed that in these latter cases some incomplete oxidations occurred. This is probably also true for *Australorbis glabratus* and *Helisoma duryi* since it is unlikely that they metabolize carbohydrate exclusively. The previously reported data concerning the respiratory quotient of *Australorbis glabratus* (von Brand, Nolan, and Mann, 1948) exclude an assumption of exclusive carbohydrate utilization, at least for this species.

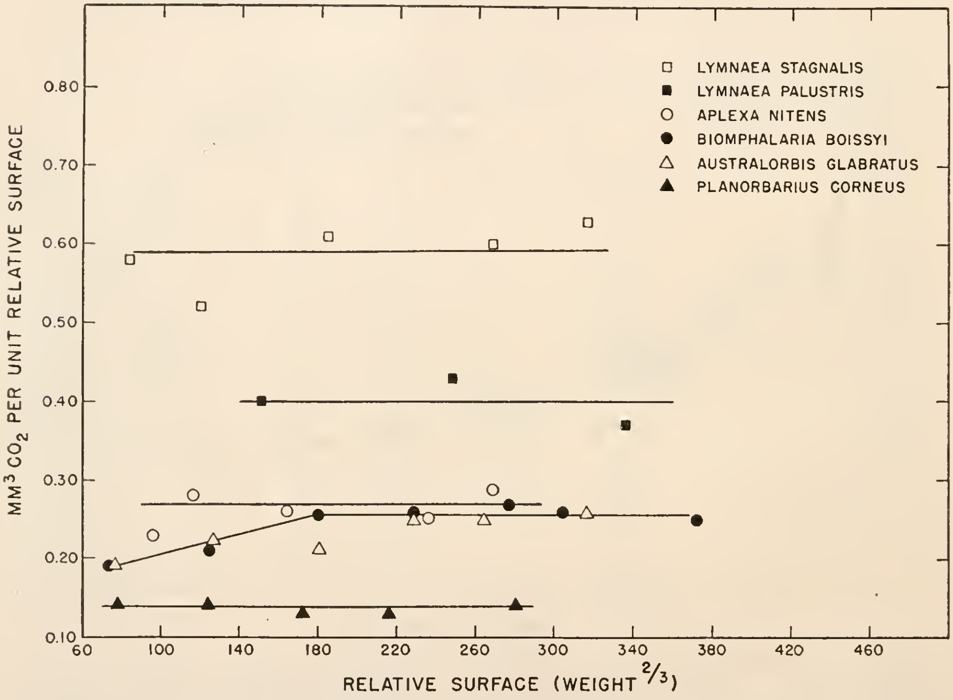

FIGURE 2. Relation between anaerobic carbon dioxide production and relative surface of pulmonate aquatic snails. The values are mean values of groups of snails varying in number between 3 and 20.

The lactic acid level of the snail tissues remained practically unchanged in most species under aerobic conditions. In *Australorbis glabratus,* however, a rather

TABLE IV

Aerobic carbohydrate metabolism of some snail species

Species	Number of experiments[1]	Experimental period, hours	Oxygen consumed mm³. per 1 gm.		Carbohydrates			Lactic acid in tissues microgram per 1 gm.		Oxygen required for total oxidation of consumed carbohydrates in per cent of oxygen consumed	Ratio $\frac{\text{anaerobic}}{\text{aerobic}}$ carbohydrate consumption
					Per cent in tissues		Mg. consumed per 1 gm. in 1 hour				
			Total period	1 hour	Initial	Final		Initial	Final		
Australorbis glabratus	18	16	2544 ±136	159	2.69 ±0.18	2.35 ±0.14	0.21	232 ±21	65 ±22	99	1.5
Helisoma duryi	29	24	1937 ±105	81	2.08 ±0.18	1.81 ±0.29	0.11	133 ±26	114 ± 9	104	4.1
Planorbarius corneus	18	24	2870 ±165	120	2.94 ±0.02	2.89 ±0.28	0.02	267 ±66	248 ±41	13	2.4
Lymnaea stagnalis	18	6	1230 ± 89	205	1.26 ±0.10	1.04 ±0.10	0.37	247 ±47	179 ±42	133	1.2
Lymnaea palustris	18	6	821 ±193	137	1.22 ±0.13	1.00 ±0.12	0.37	112 ±16	103 ±14	200	2.3
Pomatiopsis lapidaria	18	24	2230 ±108	93	1.47 ±0.08	1.37 ±0.08				30	4.5
Melanoides tuberculatus	18	24	1285 ± 90	54	2.73 ±0.13	2.46 ±0.16	0.11	89 ±20	98 ±33	156	1.4

sharp decline in lactic acid level occurred during the period of observation. No reason for this aberrant behavior can be given at the present time.

DISCUSSION

The fact that aquatic snails possess a certain tolerance towards the lack of oxygen has been known for some time. Alsterberg (1930) found the operculates Bulinus sp. and Vivipara sp. more resistant than the pulmonates *Lymnaea ovata, Lymnaea stagnalis,* and *Lymnaea truncatula. Planorbarius corneus* was only slightly more resistant than the lymnaeids. On the other hand, Raffy and Fischer (1933) reported Planorbarius was much more resistant than Lymnaea upon total immersion into oxygen-poor water. The present results, carried out under completely anaerobic conditions, are more in agreement with the latter than the former. In our experiments the Lymnaeidae and Physidae were considerably less resistant than the Planorbidae or the different operculates tested.

The anaerobic metabolic level of the nonresistant species, as expressed by the rates of CO_2 production and carbohydrate consumption, was, in general, somewhat higher than that of the resistant species, but a certain overlapping occurred. It is dubious, therefore, whether the metabolic level as such has a decisive bearing on the question of what factors are responsible for the differential sensitivity towards lack of oxygen. The metabolic level of snails depends, at a given temperature, on the nutritional state. Whether this last factor materially influences the length of anaerobic survival remains to be investigated.

A well defined difference between resistant and nonresistant species was found in respect to lactic acid. No accumulation whatever occurred during anaerobic periods in the tissues of the former; they excreted all the new-formed and possibly even some of the preformed lactic acid. On the contrary, the lactic acid level in the tissues of the nonresistant species increased rather sharply; these species were capable of excreting only part of the newly formed acid. How far this accumulation of lactic acid within the tissues was responsible for the early death of the animals cannot be decided at the present time. It is hardly likely that it was the only factor involved. It must be remembered in this connection that those species which do not show such an accumulation of lactic acid ultimately die of asphyxiation.

All species consumed carbohydrate anaerobically at a somewhat faster rate than under aerobic conditions, but only in *Helisoma duryi* and *Pomatiopsis lapidaria* was the quotient $\frac{\text{anaerobic consumption}}{\text{aerobic consumption}}$ as large as that in most hitherto-studied free-living invertebrates (older literature in von Brand, 1946; newer data in Cleary, 1948). In the other species the quotient was small, in *Australorbis glabratus, Lymnaea stagnalis,* and *Melanoides tuberculatus* as small, indeed, as in parasitic worms. It is difficult to adduce definite reasons for this rather curious observation. A low carbohydrate consumption quotient may result under anaerobic conditions, if the snail survives by producing a relatively small amount of energy; then the anaerobic carbohydrate consumption may proceed at a relatively low rate even in comparison with the much more efficient oxidative breakdown. It seems very probable that such a reduction in energy production is involved, at least to some degree, in the present case. It is quite certain that these snails possess only a tolerance towards anaerobiosis, but are not capable of leading an anaerobic life in the true sense of the word.

However, a second factor may also be involved. One of the reasons why the above carbohydrate quotient is low in many parasitic worms is the fact that they have a metabolism characterized by aerobic fermentations, which are less efficient in energy production than completely aerobic respiration and therefore increase the aerobic carbohydrate consumption, thus lowering the quotient. Our findings concerning the quantitative relationships between oxygen and carbohydrate consumption seem to indicate that such a situation may also prevail in some snails. This point will have to be clarified by further work.

It has been previously pointed out (von Brand, Nolan, and Mann, 1948) that the aerobic metabolic levels of pulmonate and operculate snails are different, but that the oxygen consumption follows the surface law both in intraspecific and interspecific comparisons. The present study reveals that, at least, in pulmonate snails (no suitable material of operculates was available), the anaerobic carbon dioxide production follows the surface law in intraspecific comparison. This seems to be the first instance that such a relationship has been established for the anaerobic metabolism of invertebrates. The same relationship, however, definitely does not hold in all cases of interspecific comparison. The reasons for differences in anaerobic carbon dioxide excretion by various species of snails may be different. Our observations on the behavior of lactic acid revealed considerable differences between species. In Lymnaeidae and Physidae this acid accounted for a rather large percentage of the excreted carbon dioxide, in other species only for a small one. It is then quite possible that different metabolic processes lead to different rates of carbon dioxide production depending upon the proportions of inorganically and organically derived carbon dioxide. Similar considerations apply to the rates of anaerobic carbohydrate consumption, various fermentative pathways liberating different amounts of energy. Whether or not fixed relationships exist between rates of anaerobic metabolism and size of snails belonging to different species, can probably be determined only after the actual energy production of the various processes has been elucidated.

A rather interesting result of our experiments is the observation that lactic acid, although produced by all snails, accounts in several species only for a small fraction of the anaerobically consumed carbohydrate; while in others it obviously represents a major end product. Snails had never before been studied in this respect, but similar differences are well known to occur in parasitic worms (review of the literature in Bueding, 1949), protozoa (literature in von Brand, 1950), as well as mollusks other than snails. Dugal and Fortier (1941) found no anaerobic lactic acid production in oysters, nor did Wernstedt (1944) in Dreissensia; while the production of rather large amounts of lactic acid have been reported in the case of Venus (Dugal, 1939). Recently, however, Humphrey (1949) observed some lactic acid production also in oysters.

Our studies indicate that the consumption of carbohydrate is probably of major importance in allowing snails to survive the adverse condition of lack of oxygen. It would then seem justifiable to attempt the destruction of snails that are present in anaerobic or semi-anaerobic habitats, by the application to such habitats of chemical compounds known to interfere with the glycolytic enzymes.

SUMMARY

1. Lymnaeidae and Physidae tolerated complete lack of oxygen less well than Planorbidae or operculates belonging to different families.

2. All species consumed carbohydrate under anaerobic conditions and produced carbon dioxide and lactic acid. While in several species the lactic acid produced was sufficient to account for all or a large part of the carbon dioxide as liberated from bicarbonate, this was not the case in other species.

3. The anaerobic metabolic level as measured by carbon dioxide production and carbohydrate consumption of the resistant species was, on an average, lower than that of the nonresistant ones. The former did not accumulate lactic acid within their tissues during an anaerobic period, while the latter did so to a marked degree.

4. In most species the anaerobic carbohydrate consumption was only slightly higher than the aerobic rate. One of the reasons for this may be the probable occurrence of aerobic fermentations in these species.

5. Lactic acid was quantitatively a major end product of the anaerobic carbohydrate consumption only in *Lymnaea stagnalis* and *Lymnaea natalensis;* in all other species unidentified end products must have prevailed.

6. The anaerobic carbon dioxide production followed the surface law in intraspecific comparisons in pulmonates rather closely, but definitely not in all cases of interspecific comparisons.

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AN ANALYSIS OF THE EFFECTS OF A SACCULINID PARASITE ON THE EXTERNAL MORPHOLOGY OF CALLINECTES SAPIDUS RATHBUN

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INTRODUCTION

There is no decapod crustacean that has been more intensively studied by American zoologists than the common and commercially valuable blue crab, *Callinectes sapidus* Rathbun. Accordingly, when we find such a well-known species infested with a rhizocephalan parasite an analysis of the effects of this type of parasitism is greatly facilitated through knowledge already available regarding the normal crab. On the other hand, it also happens that an understanding of the abnormal may shed additional light on the normal. The problem, therefore, of the relation of the parasite *Loxothylacus texanus* Boschma to the blue crab has been undertaken primarily to increase knowledge of the nature and effects of sacculinization, but also with the hope of contributing, if possible, to a better understanding of the normal host organism itself.

Much has been written concerning host-parasite relationships in the Sacculinidae and the general problem of "parasitic castration." Giard (1886, 1887), who coined the latter term, was the first to point out that male crabs infested with Sacculina often develop in a female direction as shown by changes in the form of the abdomen, the claws, and the pleopods, while the testes become more or less atrophied. Parasitized females, on the other hand, show little or no external changes. Other important observations on external modifications produced by sacculinids on their hosts were reported by Smith (1906, 1910), Potts (1910), Pérez (1933), Day (1935), Okada and Miyashita (1935), Bulgurkov (1938), and Veillet (1945). In general, it may be said that various species of sacculinids differ with respect to the extent of modifications they induce in their hosts, and variations also appear when one compares different species of hosts infested by the same parasite. A fairly complete bibliography of the literature on parasitic castration may be found in Hanström (1939) and Brown (1944).

The parasite reported on here is structurally similar to Sacculina; in fact, Boschma (1928) established the genus Loxothylacus for certain species of Rhizocephala that were included up to that time in the genus Sacculina. *Loxothylacus texanus,* described by Boschma in 1933, has been reported only from the region of the Gulf of Mexico where it parasitizes *Callinectes marginatus* (A. Milne-Edwards) as well as *C. sapidus* Rathbun.

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MATERIALS EXAMINED

Thirty parasitized crabs of the species *C. sapidus* were used in this study, with both sexes almost equally represented. The parasite is found attached to the underside of the abdomen of the host (Fig. 1), with the stalk of attachment fastened to the midline of the fourth or fifth abdominal segment, rarely on the third. As a rule, one parasite occurs on a single host, but instances of multiple infestation are not uncommon. Of 30 infested Callinectes, 18 bore one parasite, 7 had 2 parasites, 3 were triply infested, and 2 had 4 parasites apiece. The locality and collection data are as follows:

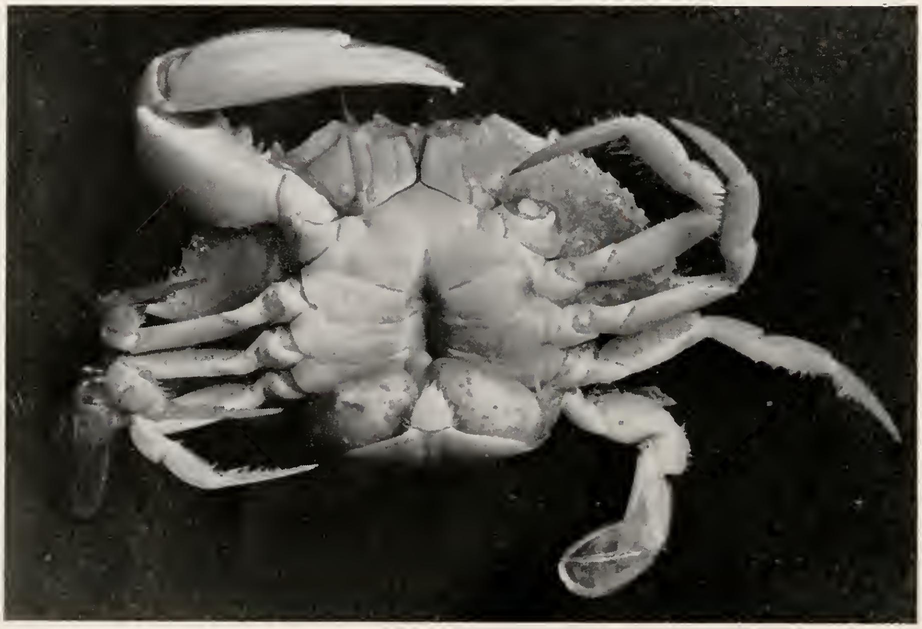

FIGURE 1. The sacculinid *Loxothylacus texanus* attached to the underside of the abdomen of *Callinectes sapidus*. The crab is an immature female of 52 mm. carapace width, but in the shape of the abdomen it resembles a normal adult female.

Florida: Cedar Keys, Oct.–Nov., 1947, 5 specimens on 2 crabs, E. Lowe Pierce coll. U.S.N.M. 89223.

Texas: Galveston Bay, April 16, 1938, 3 specimens on 2 crabs, W. G. Hewatt coll. U.S.N.M. 80465. Cedar Bayou, Nov. 14, 1947, 6 specimens on 4 crabs, Joel W. Hedgpeth coll. Aransas Bay, April, 1942, 4 specimens on 2 crabs, Gordon Gunter coll. Aransas Bay, May–July, 1948, 31 specimens on 20 crabs, Game, Fish and Oyster Commission, Rockport, Texas.

Linear measurements of the carapaces of the crabs were made according to the method presented by Gray and Newcombe (1938). Using the width as the basic index, the sizes ranged in millimeters from 38 × 19 to 75 × 35 for males, and from 40 × 18 to 85 × 38 for females. About forty normal specimens of Callinectes, selected to give the same size range as the parasitized crabs, were studied for purposes of comparison.

Effect of the Parasite on the Host

The amount of change effected by a rhizocephalan parasite in the secondary sex characters of the host appears to be correlated with the degree of differences existing between the normal males and females. Where the differences between the sexes are small, the amount of modification is also slight. *Callinectes sapidus* displays a high degree of sexual dimorphism and, correspondingly, the changes resulting from parasitism are very great. Normal male and female blue crabs are easily distinguished by the shape of the abdomen and the nature of the abdominal appendages. The adult female abdomen is broad and semicircular in outline and is composed of six distinct segments. The male abdomen has a narrow, "T"-shaped appearance, with only four of the six segments distinct, due to the fact that segments 3, 4, and 5 have fused. The pleopods of the female are biramous appendages with the exopodite slightly shorter than the endopodite. They occur on the second, third, fourth and fifth abdominal segments and the endopodite particularly is furnished with long hairs to which the eggs are attached in ovigerous specimens. The abdominal appendages of the male consist of two pairs of copulatory styles, the second being inserted into the first for the purpose of sperm transfer. Juvenile specimens are distinguishable as to sex on the same basis as the adults except that the abdomen of the immature female lacks the distinctive breadth of the adult and is triangular in shape. Further details regarding the external differences between the sexes may be found in the papers of Churchill (1919), Cochran (1935), Truitt (1939), Cronin (1947), and others.

Loxothylacus texanus evidently attacks the smaller crabs that have not yet reached maturity. The parasitized crabs varied in size from 38 mm. to 85 mm. carapace width, which is considerably below the minimal size (140 mm. carapace width) given for sexually mature Callinectes by Gray and Newcombe (1938) and Cronin (1947).

A histological study of the gonads of the parasitized crabs was not considered worth while because of the immaturity of the specimens and the absence of controls from the same locality. However, dissection of a representative sample of normal and infested crabs of comparable carapace widths clearly indicated that the gonads of the parasitized specimens were of much smaller size.

Modification of the Male Host

The most pronounced change in the infested male is the broadening of the abdomen to such a degree that it closely resembles that of a normal female (Fig. 2). This type of modification is perhaps the one most commonly noted by previous writers on the effects of sacculinization on Brachyuran crabs (Giard, 1887; Smith, 1906; Potts, 1910; Pérez, 1933; Day, 1935; Okada and Miyashita, 1935; Bulgurkov, 1938; Veillet, 1945). Measurements of the extent of modification based upon a comparison between the width of the carapace and the width of the abdomen yielded no evidence that either the size of the parasite or the number of parasites per host was related to amount of deviation from the normal. Moreover, there seemed to be no relationship evident between the degree of modification and the actual size of the host.

Broadening of the parasitized male abdomen is accompanied by an increase in the number of hairs around the margins. In normal male Callinectes the marginal hairs are more or less confined to the first four segments of the abdomen, but in the majority of parasitized specimens they are, as in the normal female, present on all the segments, forming an uninterrupted fringe. Pérez (1933) likewise noted a feminine type of villosity in sacculinized male Pachygrapsus.

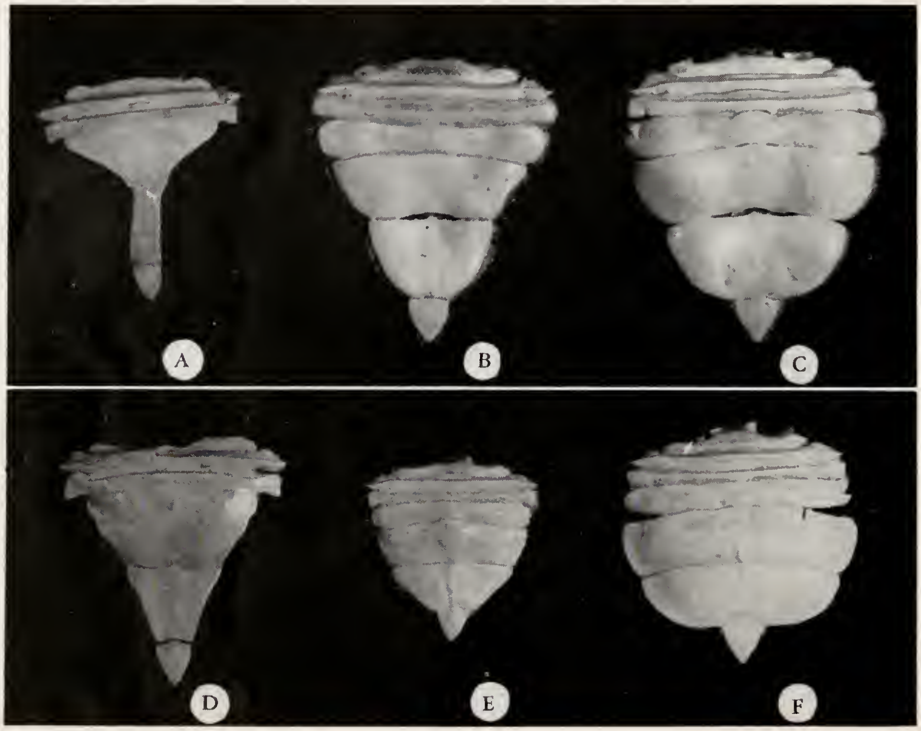

FIGURE 2. Abdomens of normal and parasitized *Callinectes sapidus.*
A. Normal male. B, C. Males parasitized by Loxothylacus. D. Normal immature female. E, F. Immature females parasitized by Loxothylacus.

Parasitism of the male also brings about complete segmentation of the abdomen. The third, fourth and fifth abdominal segments, previously coalesced, now become distinct and freely movable. Giard (1887) made a similar observation regarding *Carcinus moenas* and *Portunus holsatus* infested by sacculinids, and Potts (1910) and Veillet (1945) confirmed this in Carcinus. Day (1935) restudied the case of Portunus and showed that redivision of the male abdominal segments varied in degree, with the maximum extent more liable to occur in the smaller crabs.

The difference in abdominal segmentation between the sexes in Callinectes, as Cochran (1935) has shown, is also associated with a difference in dorsal musculature. In the female each abdominal somite has a pair of dorsal muscles, but in the male the fused section has only two pairs of dorsal muscles.

Dissection of the parasitized males, to discover whether or not total segmentation of the abdomen affects the dorsal musculature, revealed that each abdominal segment was equipped with a pair of dorsal muscles exactly as in the female (Fig. 3). Evidently a new pair of muscles arises in the previously fused region so that the three segments into which it becomes divided are each furnished with a separate pair of dorsal muscles. We are of the opinion that it is the muscles of the third somite which arise *de novo,* because the area which becomes the third somite is originally without trace of dorsal muscles, while the two pairs of existing muscles lie in the center and at the posterior region respectively of the fused section.

Turning now to the appendages of the male abdomen we find further evidence of feminization in the case of the second pleopod. This appendage is constructed on the same basic plan as the first pleopod but is much smaller and weaker. It consists of three parts (Fig. 4E): a stout, flat coxopodite; a small basipodite; and a thin end-piece usually spoken of as the "flagellum." This terminal prong is normally kept inserted in the posterior foramen of the first pleopod since its function is to force spermatophores through the much longer tube-like "flagellum" of the first pleopod which is the primary intromittent organ.

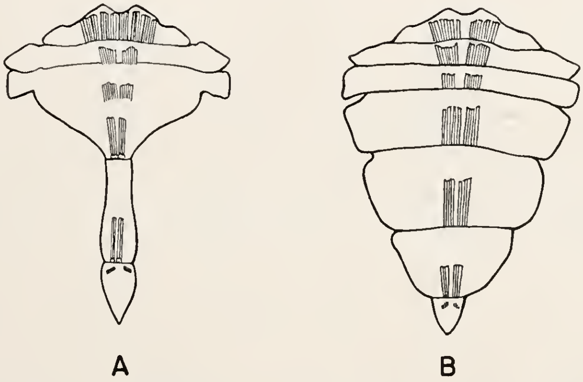

FIGURE 3. Effects of sacculinization on the segmentation and musculature of the male abdomen.

A. Abdomen of normal male Callinectes showing dorsal muscles and fusion of segments 3, 4 and 5. B. Abdomen of male parasitized by Loxothylacus. Its musculature and segmentation are like those of a normal female.

In parasitized specimens the second pleopod was never found inserted into the first and in every case its structure showed varying degrees of modification. A slightly modified type (Fig. 4F) is characterized by a shortening of the coxopodite and the bending of the "flagellum" away from the protopod so that the appendage changes from V-shaped to L-shaped in appearance. Further modification results in the condition shown in Figure 4G. Here the coxopodite has grown still smaller but the basipodite has enlarged. The terminal "flagellum" has turned still farther to the rear and its surface, previously smooth, is now furnished with short hairs.

Two of the parasitized males showed the extreme modification pictured in Figure 4I. Here the pleopod has become biramous and possesses a well-developed expodite complete even to the presence of long plumose hairs. The "flagellum" remains as the endopodite, and the entire appendage, while not identical in appearance with that of a normal female, is certainly indistinguishable from the pleopod of a parasitized female.

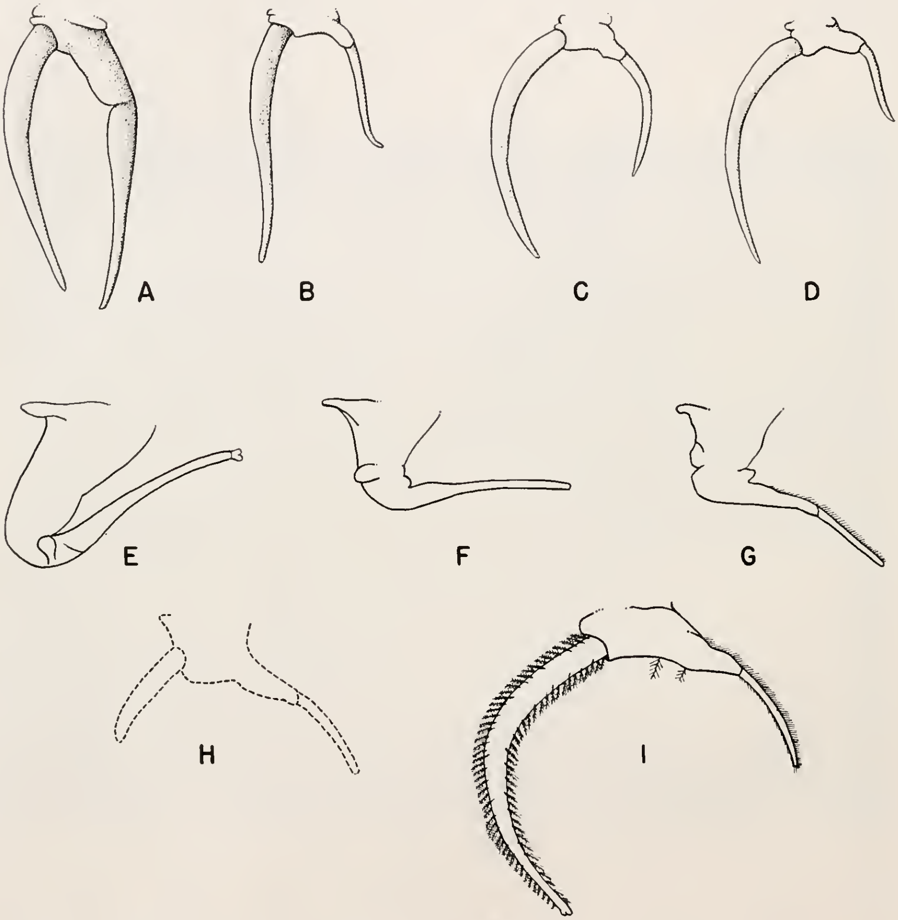

FIGURE 4. Pleopods of normal and parasitized specimens of *Callinectes sapidus*.

A. Normal female. B, C, D. Examples of female pleopods showing effects of parasitism by Loxothylacus. E. Second abdominal appendage of normal male. F, G. Minor modifications of second male pleopod resulting from parasitism. H. Hypothetical stage in transformation of male pleopod into a female type of appendage. I. Second pleopod of parasitized male showing extreme modification. This type of appendage is indistinguishable from that of a parasitized female.

Intermediate stages between Figure 4G and Figure 4I were not found. It is very probable that such do not exist, in which case one must assume that the expodite appears suddenly, following a particular molt, and is from the start long and well developed. The other alternative, that it requires more than one instar to produce a fully developed exopodite, is unlikely since Brachyuran crabs infested with sacculinids do not molt, as a rule, after the parasite has become external.

A final observation with respect to parasitized males needs to be mentioned, namely, that the concavity of the sternal plastron of the thorax is appreciably diminished, and the tubercles on the fifth sternal segment, which form a locking device to hold the abdomen flatly against the underside of the body, are greatly reduced if not entirely absent. These are both changes towards the female condition. The male gonopore is also reduced in size.

Modification of the Female Host

Female Callinectes parasitized by Loxothylacus show less extensive changes than the males. The most striking modification is that the abdomen becomes proportionally as broad or even broader than that of the normal adult female, although the size of the crab is such that normal specimens of that carapace width would still have the immature triangular abdomen (Fig. 2D–F). Of the 13 parasitized females, 8 were less than 60 mm. in carapace width (40, 42, 47, 48, 52, 52, 53 and 58 mm. respectively) and all were under 85 mm., yet each possessed a broad abdomen. The change from the triangular-shaped abdomen to a broad and rounded form does not take place in normal *Callinectes sapidus* until a carapace width of about 95–100 mm. has been reached (Newcombe, Sandoz and Rogers-Talbert, 1949). Females of that size are ready for the final molt which will reveal the form of the mature crab. Thereafter, females apparently do not molt (Churchill, 1919; Gray and Newcombe, 1939). It appears highly probable that in parasitized specimens the change in type of abdomen is likewise achieved at a single molt, but at a much earlier instar. The change is perhaps effected soon after the parasite has become established.

This precocious alteration in the shape of the abdomen of small parasitized females is a phenomenon which cannot be disregarded in attempts to explain the mechanism of parasitic castration. It parallels the cases of *Inachus mauretanicus* reported by Smith (1910) and of *Pachygrapsus marmoratus* reported by Pérez (1933), where the immature sacculinized females likewise assume prematurely the adult type of abdomen.

The pleopods of infested females are also altered to a greater or lesser degree. This involves a reduction in size of the internal ramus together with the basipodite, as shown in Figure 4A–D, and an apparent curvature of this arm away from the outer ramus so that the appendage becomes arcuate, in place of the normal ∧-shape. The expodite, on the other hand, does not seem to be affected in any significant manner.

As in the parasitized males and normal mature females, the "locking device" on the sternum of infested females is reduced or absent.

Discussion

In the introduction to this paper it was stated that an understanding of the abnormal may shed light on the normal. A good illustration of this is the manner whereby the male copulatory appendage becomes altered into a female-like biramous pleopod. The highly specialized uniramous male appendage has been difficult to homologize with the fundamental biramous plan. Cochran (1935) acknowledged this in the following statement: "The name 'flagellum' is chosen arbitrarily for the distal part of the [male] pleopod, as it does not show the character of a true flagellum. But neither is there sufficient evidence for considering it a highly modified endopodite or exopodite." Snodgrass (1936) used the term "flagellum" or telopodite for this portion of the pleopod and Cronin (1947) has employed the same terminology. That the so-called flagellum is actually a modified endopodite is clear from the transformation which the second male pleopod undergoes in parasitized crabs (Fig. 4E–I). Reconstruction of a biramous type is achieved by a completely new outgrowth, the exopodite, while the original ramus remains as the endopodite.

The parasitized crab can help us solve another morphological puzzle, namely, how to homologize the two pairs of dorsal muscles in the fused section of the normal male abdomen with those of the female. The changes in musculature which the infected male undergoes make it probable that it is the third pair which is lacking in the normal male, and therefore the designations for the muscles of the male, as given by Cochran (1935), could be made more precise.

When we analyze the entire complex of changes imposed upon the male crab by parasitism, we cannot avoid the conclusion that these modifications are all manifestations of one primary process, namely, assumption of an intersexual condition or partial feminization. The change of the male abdomen from a state of partial rigidity to one of pliability, the effects of parasitism on the concavity of the sternal plastron and on the tubercles of the fifth sternal segment, the change in structure of the second pleopod and the increase in number of hairs around the margins of the abdomen, all these are feminine characteristics.

But the female Callinectes infested with Loxothylacus shows no approach towards the opposite sex. The abdomen does not become masculine in form, rather it responds to parasitism by becoming still broader. The female pleopods, on the other hand, neither assume the adult form concomitant with the change in shape of the abdomen, nor approach the condition in the male. In the normal male, the outer ramus is absent but in parasitized females it is the inner ramus that is atrophied.

Of the various theories that have been proposed to explain the nature and effects of sacculinization, we believe that Goldschmidt's theory (1931) fits the case presented here better than any of the others. Goldschmidt postulated that the quantitative balance between male and female sex substances present as a result of genic action in each individual was disturbed by the parasite, resulting in the characteristic modification. This explanation is based on his "theory of intersexuality." An individual becomes purely male or female when the substance produced by the M-gene or the substance produced by the F-gene exceeds the opposite by a certain quantitative value. In case the inequality between the two substances is not great enough to surpass the threshold, varying degrees of intersexuality will result. Moreover, an individual could develop as a male or a female up to a certain point of time, then, if for some reason an imbalance in the production of M or F substances occurred, development would switch over to the opposite sex and an intersex would be produced.

Goldschmidt regarded the modifications produced by rhizocephalids on their crustacean hosts as cases of "parasitic intersexuality." This appears to hold good for the parasitized males that exhibit feminine secondary sex characters, but not for such parasitized females as Inachus, Pachygrapsus, or Callinectes which are modified but not in the direction of intersexuality. In fact, Goldschmidt, although aware of the effects of parasitism on female Inachus, disregards the modified females entirely in his interpretation of the known phenomena of sacculinization.

Despite this omission, the theory of Goldschmidt need not be altered in its essentials to cover the case of the females. If, in a genetic male, the factor of parasitism enhances the reaction due to the F-gene or depresses that induced by the M-gene to the point where the normal relation became reversed and an intersex results, why should not the same factor operating on a female likewise increase the F-reaction or depress the M-reaction? The result in the latter case would be not intersexuality but a "strengthening" of femaleness. This reasoning provides a plausible explanation for sacculinized Inachus, Pachygrapsus and Callinectes insofar as the precocious development of certain adult characteristics in the immature female is concerned.

Exactly how the abnormal balance in the sex substances of the host is achieved by action of the parasite is a question difficult to answer. Reverberi (1944–45), who also made use of Goldschmidt's theory to explain the complex phenomena of parasitic castration, presents the suggestion that the parasite itself might elaborate a substance similar to F or activate in the host a superproduction of the F-substance; or conversely, it might take away the M-substance from the host or inhibit the production of the M-substance. Admittedly this is purely conjectural, but certainly within the realm of possibility.

Goldschmidt's theory, modified in the way in which we have just outlined it, appears to explain all the changes observed in parasitized Callinectes except for the strangely altered pleopods of the female. We shall now attempt to account for this exception.

First of all, the morphological nature of the altered female pleopods is such that one cannot describe them as retarded in development nor, strictly speaking, as approaching the normal male type. Neither can pressure against them by the external sac of the parasite explain their deformation, since various authors (Giard, 1887; Smith, 1906; Bulgurkov, 1938) have shown that sacculinized female crabs may display deformed swimmerets before the parasite becomes external.

Secondly, it is important to realize that the entire pleopod does not atrophy; the affected portion is the endopodite. The function of this ramus in normal crabs is an ovigerous one, while the external ramus is not concerned with the carrying of eggs. For this reason the endopodite may be regarded as a special type of accessory female sex structure whose normal development may be causally connected with the proper functioning of the ovary.

Le Roux (1931, 1931a) has shown that in female Gammarus the differentiation of certain secondary sex characters is determined by the functioning of the gonads, while that of others evolves independently of them. These results are in accord with those obtained by Haemmerli-Boveri (1926) on the females of the isopod Asellus, and by Callan (1940) on female prawns of the genus Leander. It is highly probable, therefore, that there exists in crustaceans an ovarian hormone which controls the differentiation of certain structures associated with the care of developing young.

If then, in Callinectes, the differentiation of the inner rami of the pleopods is under the special influence of the gonads, atrophy of the sex glands as a result of parasitism would explain the failure of the endopodites to develop properly;[2] whereas the abdomen, whose morphogenesis may have no causal connection with this ovarian hormone, would evolve in a manner determined by the amount of F-substance present at a particular period of development.

Postulation of a feminizing stimulus elaborated by the parasite would not be contrary to the view maintained by us in an earlier paper, (Reinhard and von Brand, 1944) regarding toxic substances. Toxic agents could still be responsible for the atrophy of gonads and various physiological disturbances of the host. But the external morphological changes, which appear to operate chiefly in the same direction in both sexes, could well be determined in the manner just outlined.

Summary and Conclusions

Loxothylacus texanus Boschma, a rhizocephalid of the family Sacculinidae, infests the blue crab, *Callinectes sapidus* Rathbun, in the region of the Gulf of Mexico and causes alternation of the secondary sex characters of the host. The parasite attacks the smaller crabs that have not yet attained sexual maturity.

In accordance with the high degree of sexual dimorphism of the host species, the effects on the male are most striking since they tend to make it resemble the female. The narrow abdomen becomes broad and marginal hairs are increased. The third, fourth and fifth abdominal segments, previously fused, become freely movable; while the dorsal abdominal musculature comes to correspond with the altered segmentation. The second male pleopod shows varying degrees of modification resulting in two instances in a biramous type of appendage. The concavity of the sternal plastron, the tubercles on the fifth segment, and the gonopore are reduced.

In the immature female host, the triangular abdomen also assumes the broadness of the normal adult female, the sternal tubercles atrophy and the pleopods are altered to an arcuate form through curvature and shortening of the endopodite. No approach towards the opposite sex takes place in parasitized females.

The external modifications in parasitized male Callinectes clearly fall into the category of "parasitic intersexuality" and may be interpreted as resulting from a disturbance of the balance between male and female sex-formative substances brought about by the action of the parasite. The same type of parasitic activity, operating on a female host, should likewise increase the relative amount of female substance. Therefore immature parasitized Callinectes, instead of becoming intersexual, are hyperfeminized and develop precociously the adult female type of abdomen. The female pleopods, however, may constitute a special type of secondary sex character independent of the sex substances but dependent upon the normal maturation of the ovary. Suppression of ovarian growth by the parasite would then interfere with their proper development.

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[2] Another instance of this is the case of *Pagurus longicarpus* recently reported by the writer (Reinhard, 1950, *Journ. Parasit.*, **36** (2): 131–138) where the chief effect of parasitism by an entoniscid is the suppression of the normal development of the endopodites of the female crab.

These two-fold agencies, an upset in the normal relationships between the male and female sex-formative substances and the loss of ovarian control over the differentiation of the female pleopods, together give a plausible explanation of the external effects of sacculinization, particularly when applied to the case of Callinectes parasitized by Loxothylacus.

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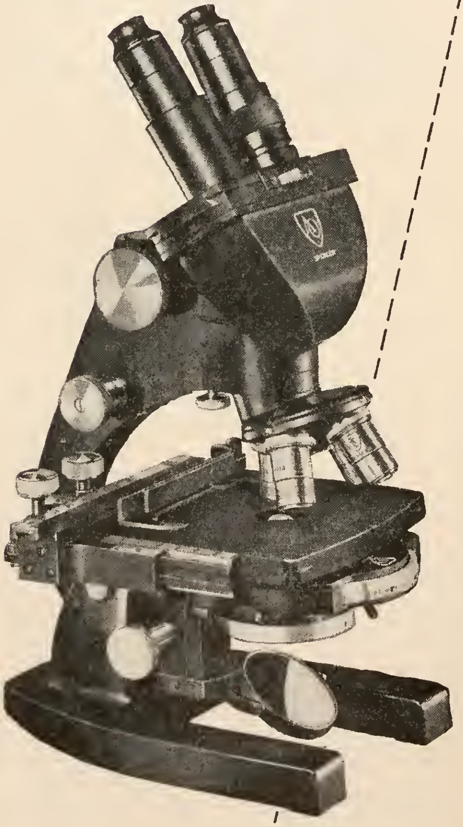

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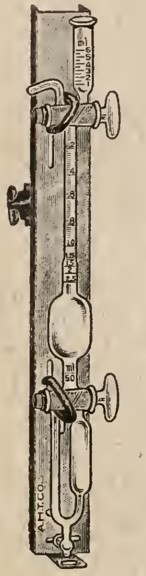

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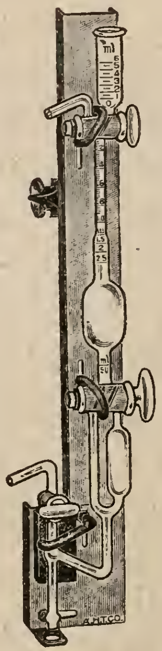

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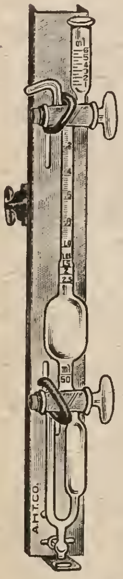

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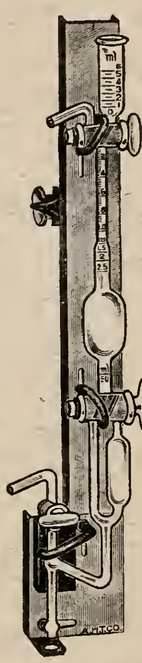

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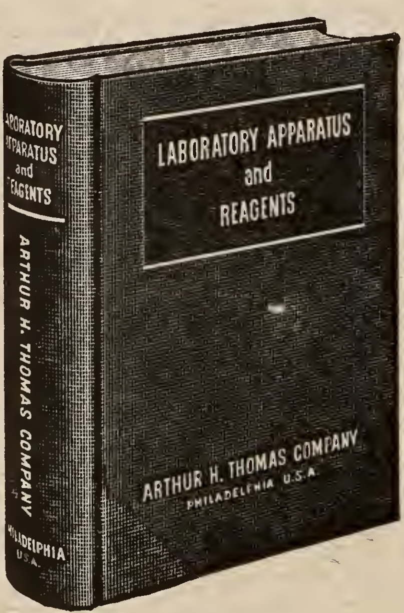

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